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Pathways of Pyruvate Metabolism in the Mammary Gland*

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(*Received* 10 *March* 1951)

The utilization of glucose and acetate by lactating mammary tissue *in vitro* has been demonstrated by Folley & French (1949*a*, 1950). The observation that these substrates are metabolized with a high respiratory quotient is regarded as evidence for their utilization in milk-fat synthesis.

In the present paper an account is given of experiments on the metabolism of pyruvate in mammary tissue. The evidence obtained suggests that the Krebs citric acid cycle is operative in the mammary gland. However, only a fraction of the pyruvate metabolized is oxidized completely and a large part appears to be utilized in synthetic reactions, a conclusion supported by the use of 2:4-dinitrophenol (DNP), which is known to inhibit synthetic reactions by interfering with the transfer of phosphate-bond energy generated by the Krebs cycle (Lipmann, 1946; Loomis & Lipmann, 1948).

EXPERIMENTAL

Material. Mammary gland tissue of lactating rats, rabbits, or goats was removed immediately after the death of the animal. Sheep mammary tissue was obtained by biopsy under *cyclo*propane anaesthesia. Tissue slices, cut by the method of Stadie & Riggs (1944), were shaken in three changes of ice-cold saline to remove as much milk as possible; they were then blotted and 100–150 mg. portions weighed on a torsion balance and placed in the prepared media in Warburg flasks. These slices will be referred to as 'non-depleted' or 'untreated' slices.

'Depleted' tissue. In order to reduce their stores of preformed substrates, the slices were subjected in bulk to a preliminary incubation for 1 hr. at 37° in a 100 ml. measuring cylinder containing 50 ml. of saline through which a rapid stream of O_2 was passed. The tissue was then again shaken in ice-cold saline, blotted, and weighed portions transferred to Warburg flasks. 'Depleted' slices were used in all experiments described in this paper, unless otherwise stated.

Dry weights. At the end of the experimental period the cups were cooled in ice water and HCl added (final concn. 0·04 N); the tissue portions were removed from the media, washed in distilled water and dried in a steam oven overnight. All metabolic quotients were calculated from the final dry weight of the tissue. The ratio final dry weight/initial wet weight was 0·14–0·15 for lactating mammary gland slices, and 0·4–0·5 for pregnant mammary gland slices.

* Preliminary communication, Terner (1950).

Saline media. Phosphate saline without calcium (Krebs & Eggleston, 1940) was the standard medium. The bicarbonate saline of Krebs & Henseleit (1932) was used in some experiments.

Analytical methods. Pyruvate was estimated by the method of Friedemann & Haugen (1943). The method involving extraction with benzene or toluene gave the same results as the direct method ('total hydrazones') and the latter was in a few determinations used for greater speed. The period of incubation of the sample with 2:4-dinitrophenylhydrazine was 5 min. in all cases.

Citric acid was determined by the method of Weil-Malherbe & Bone (1949), acetoacetic acid according to Krebs & Eggleston (1945), lactic acid by the method of Barker & Summerson (1941) and glucose by the method of Nelson (1944). For the estimation of 'total bound carbohydrate' the tissue in its medium (after withdrawal of a 0·5 ml. sample for pyruvate estimation) was hydrolysed with N-KOH at 100° for 30 min., followed by hydrolysis in N-H_2SO_4 at 100° for 180 min. Reducing sugar was then determined by the method of Nelson (1944) and calculated as glucose. For the determination of steam-volatile fatty acids, 1–2 g. portions of depleted mammary gland slices were incubated in large manometric flasks (Krebs & Eggleston, 1945) containing 20 ml. of saline and added substrates. At the end of the incubation period, 5 ml. of 2 N-H_2SO_4 were added and the tissue removed. The media were deproteinized with tungstic acid and steam distilled in the apparatus described by Markham (1942); this was followed by redistillation from HgO according to Friedemann (1938). The distillates were analysed by liquid-gas partition chromatography (James & Martin, 1951). These analyses were kindly carried out by Dr A. J. P. Martin.

R.Q. was determined by the method of Dickens & Šimer (1931), using Dickens & Greville (1933) flasks.

A diluted solution of pyruvic acid was kept as a stock solution and portions were neutralized before each experiment as recommended by Lipschitz, Potter & Elvehjem (1938).

RESULTS

Endogenous respiration and effect of fumarate. The endogenous respiration of lactating mammary tissue is high, and the increase of O_2 uptake on addition of substrates can often be relatively small. Preliminary incubation of the tissue slices without added substrates (see Experimental section) reduced the endogenous Q_{O_2} of fresh rat tissue from -4 to -8 (cf. Folley & French, 1949*a*) to -2 to -3. The depleted slices respired with high Q_{O_2} in the presence of glucose or pyruvate, but in many

Table 1. *Effect of prolonged incubation on metabolism of lactating mammary tissue*

(Rat mammary gland slices incubated in Warburg flasks at 37° after (*a*) 60 min. aerobic incubation at 37°, (*b*) 60 min. storage in ice-cold saline. Phosphate saline without calcium (Krebs & Eggleston, 1940); gas, 100% O_2; glucose, pyruvate, 0·01 M.)

Exp. no.	Tissue portion	Preliminary treatment	Substrates added	$-Q_{O_2}$ during period 30–60 min.	$-Q_{O_2}$ during period 60–120 min.
1	1		None	2·9	2·2
	2	Incubated at 37°	Glucose at 60 min.	3·1	4·1
	3		Pyruvate at 0 min.	8·3	8·5
	4	Stored at 0°	Glucose at 60 min.	6·5	8·4
				10–30 min.	30–90 min.
2	1		None	1·1	1·0
	2	Incubated at 37°	Glucose at 30 min.	1·0	1·7
	3		Pyruvate at 30 min.	0·9	1·7
	4		Pyruvate at 0 min.	10·7	10·6
	5		None	7·4	4·2
	6	Stored at 0°	Pyruvate at 0 min.	8·9	8·1
	7		Pyruvate at 30 min.	5·2	6·5

experiments the increase of respiration was small or absent, when the addition of the substrate had been delayed for 30–60 min. from the start of the incubation period in the Warburg apparatus (Table 1). The addition of fumarate (0·002–0·01 M) to the medium in the Warburg flasks restored to the depleted tissue its ability to metabolize glucose or pyruvate, even when these substrates were added after long periods of incubation (up to 2 hr.). Fumarate alone did not increase the O_2 consumption above the endogenous level (Table 2).

In order to test whether prolonged incubation without added substrates damaged the respiratory mechanism of the tissue, slices from the same gland were divided into two portions *a* and *b*. Slices *a* were incubated at 37° for 60 min., while slices *b* were stored in ice-cold saline. Subsequently the depleted slices *a* respired in the presence of fumarate and either glucose or pyruvate at approximately the same high rates as the untreated slices *b* ($Q_{O_2} - 6$ to -11; cf. Folley & French, 1949*a*), while the endogenous respiration of slices *a* had been reduced to one-third or one-half of that of slices *b* (Table 1). The depletion process, therefore, does not impair the ability of the tissue to metabolize added substrates, and increases the magnitude of substrate respiration in relation to the endogenous respiration. Oxaloacetate or a precursor is an essential metabolite which is lost from the system during prolonged preliminary incubation. Fluoride (0·005–0·01 M) was in some experiments added to untreated tissue in order to inhibit the utilization of endogenous substrates.

Effect of fumarate and malonate on metabolism of pyruvate. When depleted tissue was incubated with malonate (0·0025–0·01 M) the increase of Q_{O_2} on addition of pyruvate was inhibited. In the presence of fumarate, the inhibition due to malonate was to some extent reversed. In mammary gland slices which had not been depleted, pyruvate was oxidized in the presence of malonate at rates of the same order of magnitude as those observed in depleted tissue incubated with fumarate, malonate and pyruvate (Tables 2 and 3).

Formation of citric acid. Knodt & Petersen (1946) reported the *in vitro* formation by mammary tissue of citric acid from endogenous substrates. Added substrates increased the amount of citric acid formed, but the authors could not exclude the possibility that these substances 'acted indirectly to increase citric acid by stimulating other metabolic processes which in turn increase citric acid formation'. In the present experiments, citric acid was not found in measurable quantities after the incubation of depleted mammary gland slices without added substrates. On incubation with fumarate and pyruvate, however, small amounts of citric acid accumulated (Table 4).

The role of fumarate in the metabolism of pyruvate, the inhibitory effect of malonate, its reversal by fumarate, and the formation of citric acid during the metabolism of pyruvate are evidence suggesting the operation of the citric acid cycle in the mammary gland (see Krebs & Eggleston, 1940).

Effect of DNP on metabolism of pyruvate. In the course of a study of the effect of inhibitors on the metabolism of pyruvate in lactating mammary gland slices of the rat, rabbit and sheep, the addition of 2×10^{-4} M-DNP was found to cause a large increase of the O_2 uptake in the presence of pyruvate plus fumarate (Fig. 1). When added in the absence of pyruvate, DNP caused inhibition of the endogenous respiration (cf. Dodds & Greville, 1934). Estimation of the amount of pyruvate disappearing showed that in the absence of DNP less oxygen was taken up by the tissue slices than corresponded to the complete oxidation of the pyruvate metabolized, the ratio extra O_2 taken up/pyruvate disappearing being less than 1. On addition of DNP, the rate at which

Table 2. *Effect of fumarate and malonate on respiration of lactating mammary tissue*

(Rat mammary gland slices incubated in Warburg flasks at 37° after (*a*) 60 min. aerobic incubation at 37°, (*b*) 60 min. storage in ice-cold saline. Phosphate saline; gas, 100% O_2; pyruvate, 0·01 M; malonate, 0·01 M.)

Exp. no.	Tissue portion	Preliminary treatment	Substrates added	$-Q_{O_2}$ during period (min.) 30–60	60–120
1	1	Incubated at 37°	None	3·6	3·0
	2		Fumarate (0·005 M) at 0 min.	4·0	3·6
	3		Fumarate (0·005 M) at 0 min.; pyruvate at 60 min.	5·0	7·1
2	1	Incubated at 37°	None	2·9	2·4
	2		Pyruvate at 60 min.	2·3	4·4
	3		Fumarate (0·005 M) at 0 min.; pyruvate at 60 min.	3·0	6·4
	4		Malonate at 0 min.; pyruvate at 60 min.	2·4	3·0
	5		Fumarate (0·01 M), malonate at 0 min.; pyruvate at 60 min.	3·6	5·6
	6	Stored at 0°	None	4·3	2·9
	7		Pyruvate at 60 min.	4·3	7·5
	8		Malonate at 0 min.; pyruvate at 60 min.	3·2	5·7

Table 3. *Effect of fumarate and malonate on metabolism of pyruvate in lactating mammary tissue*

(Rat mammary gland slices incubated at 37° in phosphate saline at 37°; gas, 100% O_2. Depleted slices, except in Exp. 4. Pyruvate, 0·005 M.)

Exp. no.	Incubation period (min.)	Tissue portion	Additions	$-Q_{O_2}$	$Q_{pyruvate}$
1	100	1	None	3·5	—
		2	Fumarate (0·005 M), pyruvate	5·9	−4·3
		3	Malonate (0·005 M), pyruvate	4·8	−2·9
2	85	1	None	2·0	—
		2	Fumarate (0·002 M), pyruvate	5·3	−4·3
		3	Malonate (0·0025 M), pyruvate	2·7	−3·0
3	170	1	None	1·4	—
		2	Fumarate (0·002 M), pyruvate	4·5	−3·2
		3	Malonate (0·01 M), pyruvate	1·3	−1·1
		4	Fumarate (0·002 M), malonate (0·01 M), pyruvate	2·3	−1·8
4	110	1	Malonate (0·01 M)	1·6	—
		2	Malonate (0·01 M), fumarate (0·005 M)	1·8	—
		3	Fumarate (0·005 M), pyruvate	5·4	−3·7
		4	Malonate (0·01 M), pyruvate	4·1	−2·9
		5	Malonate (0·01 M), fumarate (0·005 M), pyruvate	4·9	−3·4

Table 4. *Formation of citric acid in mammary tissue*

(Mammary gland slices incubated at 37° in phosphate saline; gas, 100% O_2; fumarate (0·002 M) in all cups; pyruvate 0·005 M; 2:4-dinitrophenol, 2×10^{-4} M. Exp. 1, rat; Exps. 2 and 3, rabbit. Incubation period, 60 min.)

Exp. no.	Tissue portion	Additions	$-Q_{O_2}$	$Q_{pyruvate}$	$Q_{citric\ acid}$
1	1	None	2·2	—	<0·1
	2	Pyruvate	4·3	−4·1	+1·2
	3	Pyruvate, DNP	6·2	−4·5	<0·1
	4	Pyruvate, fluoroacetate (0·005 M)	5·2	−3·5	+1·1
2	1	None	3·7	—	<0·1
	2	Pyruvate	9·5	−5·8	+0·4
	3	Pyruvate	8·9	−4·9	+0·3
	4	Pyruvate, DNP	13·4	−6·0	<0·1
	5	Pyruvate, DNP	13·3	−6·1	<0·1
3	1	None	2·9	—	<0·1
	2	Pyruvate	8·4	−5·4	+1·1
	3	Pyruvate, DNP	16·0	−6·8	<0·1

pyruvate was metabolized ($Q_{pyruvate}$) was not considerably, if at all, increased, although the O_2 consumption increased by 50–100 %. The stimulation of O_2 uptake by DNP was much greater in those experiments in which the addition of pyruvate had resulted in only a small stimulation of respiration above the endogenous level. When the increase due to addition of pyruvate had been large, the effect of DNP was relatively small. The ratio extra oxygen consumed/pyruvate disappearing was increased by DNP to a value of 1·5–2, indicating that pyruvate is more completely oxidized in the presence of DNP than in its absence (Table 5). No citric acid measurable by the experimental technique employed was found after incubation in the presence of DNP. In most experiments the increase of O_2 uptake due to DNP greatly exceeded the extra O_2 consumption that might have been expected from the complete oxidation of the citric acid accumulated in the absence of DNP (Table 4).

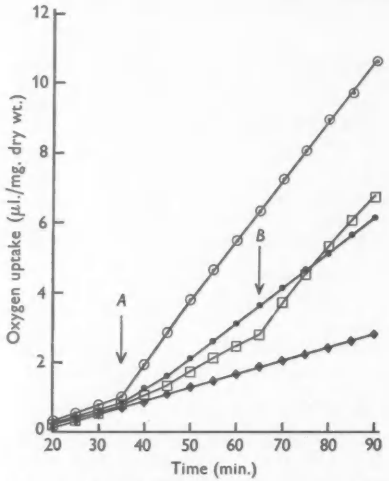

Fig. 1. Effect of 2:4-dinitrophenol on pyruvate metabolism in 'depleted' mammary tissue. Rat mammary gland slices incubated in phosphate saline at 37°; gas, 100 % O_2; 0·002 M-fumarate in all cups. ◆—◆, no addition; ●—●, pyruvate (0·01 M) added at *A*; □—□, pyruvate added at *A*, DNP (2×10^{-4} M) at *B*; ○—○, pyruvate and DNP at *A*.

Pyruvate metabolism of non-lactating mammary tissue. The largest and most regular increases in oxygen consumption on addition of DNP to mammary tissue metabolizing pyruvate were observed at the height of lactation. Mammary gland slices from pregnant rats (20th day of pregnancy), despite their low rate of respiration (Q_{O_2} about -1, cf. Folley & French, 1949*b*), showed a distinct increase of oxygen consumption on addition of pyruvate and 2×10^{-4} M-DNP (Fig. 2).

Formation of acetic acid. In view of the *in vitro* utilization of acetate by the mammary gland (Folley & French, 1950), accumulation of acetate derived from the oxidative decarboxylation of pyruvate is not to be expected. Acetate was found in small amounts in the steam distillates of the media recovered from large-scale experiments (see Experimental section); and was increased when pyruvate was present. Traces of propionic, butyric and *iso*-butyric acid found in some samples may have been

Table 5. *Effect of inhibitors on metabolism of mammary tissue*

(Rabbit mammary gland slices incubated at 37° in phosphate saline; gas, 100 % O_2; fumarate (0·002 M) in all cups; pyruvate, 0·005 M; glucose, 0·01 M; acetate, 0·01 M; fluoroacetate, 0·005 M; fluoride, 0·005 M.)

Tissue portion	Additions	$-Q_{O_2}$ during period (min.) 10–40	40–70	70–100	$-Q_{pyruvate}$ during period 40–100 min.	$-\Delta Q_{O_2}$ during period 40–100 min.	Ratio $\Delta Q_{O_2}/Q_{pyruvate}$
1	None	3·0	2·7	3·1	—	—	—
2	Pyruvate at 40 min.	3·2	6·9	10·0	5·4	5·3	1·0
3	Pyruvate at 40 min.	2·9	5·7	7·8	3·4	3·9	1·1
4	Pyruvate; DNP at 40 min.	3·0	14·7	17·3	6·9	13·0	1·9
5	Pyruvate; DNP at 40 min.	3·2	14·3	16·2	7·0	12·1	1·7
6	Glucose, acetate at 40 min.; DNP at 70 min.	3·1	10·2	16·9	—	—	—
7	Glucose, acetate at 40 min.; DNP at 70 min.	3·1	12·0	17·5	—	—	—
8	Fluoride at 0 min.; glucose, acetate at 40 min.; DNP at 70 min.	1·5	3·5	2·5	—	—	—
9	Fluoroacetate at 0 min.; pyruvate at 40 min.	1·0	1·0	1·0	1·1	—	—
10	Fluoroacetate at 0 min.; pyruvate, DNP at 40 min.	1·4	2·0	1·4	0·4	—	—
11	Pyruvate at 40 min.; fluoroacetate at 70 min.	2·8	7·0	4·4	3·6	—	—
12	Pyruvate at 40 min.; fluoroacetate at 70 min.	2·5	6·0	4·1	3·1	—	—

derived from tissue debris. The extra acetic acid accumulated in the presence of pyruvate was equivalent to 5–10% of the pyruvate metabolized (Table 6).

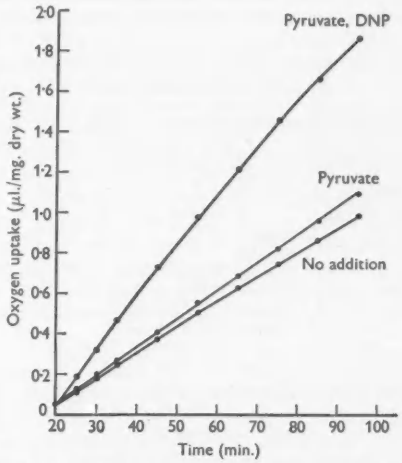

Fig. 2. Effect of 2:4-dinitrophenol on pyruvate metabolism in non-lactating mammary tissue. Rat mammary gland slices (20th day of pregnancy) (not depleted) incubated in phosphate saline at 37°. Fumarate (0·002 M) in all cups; additions: pyruvate (0·005 M), DNP (2×10^{-4} M).

Formation of lactic acid. Only small amounts of lactic acid were formed from pyruvic acid in the presence or absence of DNP (Table 7).

Formation of acetoacetic acid. Acetoacetic acid was not found in measurable amounts after incubation of mammary gland slices with fumarate and pyruvate, in the presence or absence of DNP.

Changes in tissue carbohydrate. Rat mammary gland slices washed in several changes of saline contained 0·25–0·3% of 'total bound carbohydrate' (see Methods section). On incubation without added substrates, the carbohydrate content decreased slightly, but was approximately maintained in the presence of pyruvate. Table 8 shows that the changes in carbohydrate content were small in relation to the amount of pyruvate metabolized, and that less than 10% of the pyruvate disappearing was utilized in the maintenance of the carbohydrate level. DNP appeared to inhibit the synthesis of carbohydrate from pyruvate.

Table 6. *Formation of acetic acid in mammary tissue*

(Mammary gland slices (approx. 1·5 g.) incubated at 37° in phosphate saline in large manometric flasks (Krebs & Eggleston, 1945); gas, 100% O_2; tissue of Exps. 1 and 2 not depleted; tissue of Exp. 3 depleted by preliminary incubation for 60 min.; fumarate (0·005 M) in all cups; fluoride, 0·01 M; pyruvate, 0·002 M; DNP, 5×10^{-5} M; media of Exp. 3 were saponified before steam distillation; Exps. 1 and 3, rat; Exp. 2, rabbit. Incubation period 120 min.)

Exp. no.	Additions	$-Q_{O_2}$	$-Q_{pyruvate}$	$Q_{acetic\ acid}$
1	Fluoride	1·7	—	0·2
	Fluoride, pyruvate	2·8	2·1	0·3
2	Fluoride	1·2	—	0·1
	Fluoride, pyruvate	3·0	2·8	0·1
3	None	2·1	—	0·1
	Pyruvate	3·6	3·0	0·3
	Pyruvate, DNP	5·8	3·7	0·5

Table 7. *Formation of lactic acid in mammary tissue*

(Rabbit mammary gland slices incubated at 37° in phosphate saline; gas, 100% O_2; fumarate (0·002 M) in all cups. Incubation period, 90 min.)

Exp. no.	Additions	$-Q_{O_2}$	$-Q_{pyruvate}$	$Q_{lactic\ acid}$
1	None	2·3	—	0·6
	Pyruvate (0·005 M)	4·0	5·1	0·9
	Pyruvate (0·005 M), DNP (2×10^{-4} M)	11·8	6·1	1·2
2	None	2·1	—	0·5
	Pyruvate (0·0025 M)	3·7	2·6	1·2
	Pyruvate (0·0025 M), DNP (5×10^{-5} M)	7·1	3·7	0·8

Table 8. *Changes in carbohydrate content of mammary gland slices*

(Rat mammary gland slices incubated at 37° in phosphate saline; gas, 100% O_2. Incubation period, 120 min. Pyruvate, 0·01 M, fumarate, 0·02 M, 2:4-dinitrophenol, 2×10^{-4} M. Preliminary treatment: slices washed by shaking in ice-cold saline; no preliminary incubation.)

Tissue portion	Wet wt. (mg.)	Additions	O_2 uptake (μl.)	Pyruvate disappearing (μl.)	Total bound carbohydrate as glucose (μg.)	(μl.)
1	196·5	Not incubated	—	—	585	73
2	195·0	None	127	—	504	63
3	196·5	None	99	—	450	56
4	195·5	Fumarate, pyruvate	382	354	604	75
5	197·0	Fumarate, pyruvate	305	273	571	71
6	196·0	Fumarate, pyruvate, DNP	875	452	518	65

Effect of DNP on glucose metabolism. In contrast to its stimulating effect on the oxygen uptake of mammary tissue metabolizing pyruvate, 2×10^{-4}M-DNP did not increase, and in most experiments inhibited, respiration when glucose was the substrate (Table 9). Lower concentrations of DNP (2×10^{-5}M) stimulated respiration in the presence of glucose (Table 10). This may be partly due to the stimulation by DNP of aerobic glycolysis (see Dodds & Greville, 1934). 5×10^{-5}M-DNP was variable in its effect on glucose metabolism, causing definite stimulation in some experiments, and definite inhibition in others. It therefore appears that DNP affects carbohydrate metabolism in at least two ways: (1) by influencing the glycolytic breakdown of glucose to pyruvate; (2) by influencing the pathways of pyruvate metabolism, causing the larger part of pyruvate to be oxidized through the Krebs cycle.

Table 9. *Effect of* 2:4-*dinitrophenol on metabolism of mammary tissue*

(Rat mammary gland slices incubated at 37° in phosphate saline; gas, 100% O_2; fumarate (0·002M) in all cups; pyruvate, 0·05M; glucose, 0·005M; DL-lactate, 0·005M.)

Tissue portion	Additions	$-Q_{O_2}$ during period (min.) 30–60	60–120
1	None	3·3	2·8
2	Pyruvate at 30 min.	5·6	4·8
3	Pyruvate, 2×10^{-5}M-DNP at 30 min.	5·3	4·6
4	Pyruvate, 5×10^{-5}M-DNP at 30 min.	8·7	9·0
5	Pyruvate at 30 min., 10^{-4}M-DNP at 60 min.	5·0	7·9
6	Pyruvate at 30 min., 2×10^{-4}M-DNP at 60 min.	5·7	13·5
7	Glucose at 30 min.	4·5	3·9
8	Glucose at 30 min., 2×10^{-4}M-DNP at 60 min.	4·4	2·2
9	Lactate at 30 min.	4·1	2·3
10	Lactate at 30 min., 10^{-4}M-DNP at 60 min.	4·2	3·1
11	Lactate at 30 min., 2×10^{-4}M-DNP at 60 min.	4·2	2·2

It follows that in the presence of DNP in high concentration the formation of pyruvate from glucose will be inhibited and the stimulating effect of DNP on the metabolism of pyruvate will not be apparent. It is of interest, in this connexion, that in some experiments in which acetate was present in addition to glucose, 2×10^{-4}M-DNP caused some stimulation of O_2 consumption by mammary gland slices of the rabbit (Table 5), although in most experiments Q_{O_2} was inhibited by DNP in the presence of both glucose and glucose plus acetate. It seems plausible that 2-carbon fragments derived from acetate can under suitable conditions supplement the supply of 2-carbon fragments, thus enabling the Krebs cycle to proceed at an adequate rate.

Table 10. *Effect of* 2:4-*dinitrophenol on metabolism of glucose in mammary tissue*

(Rat mammary gland slices incubated at 37° in phosphate saline; gas, 100% O_2; fumarate (0·002M) in all cups; glucose, 0·005M; incubation period 90 min.)

Tissue portion	Additions	$-Q_{O_2}$
1	None	2·6
2	Glucose	6·8
3	Glucose, 10^{-5}M-DNP	6·4
4	Glucose, 2×10^{-5}M-DNP	10·5
5	Glucose, 5×10^{-5}M-DNP	2·7
6	Glucose, 2×10^{-4}M-DNP	2·3

Effect of fluoroacetate. Bartlett & Barron (1947) found that fluoroacetate inhibits the O_2 uptake and pyruvate utilization of tissue slices (kidney, heart, liver) and of minced muscle. Similar results were obtained with mammary gland slices (Table 5). As in yeast (Kalnitsky & Barron, 1947; Black & Hutchens, 1948), inhibition of respiration and pyruvate utilization were most nearly complete when fluoroacetate (0·005–0·05M) was present before pyruvate was added. Delayed addition of fluoroacetate often resulted in 30–50% inhibition of pyruvate utilization and respiration. In other experiments, fluoroacetate (0·01–0·025M) did not inhibit the oxygen uptake of mammary tissue metabolizing pyruvate. According to Bartlett & Barron

Table 11. *Effect of fluoroacetate on pyruvate metabolism in mammary tissue*

(Rat mammary gland slices incubated in phosphate saline at 37°; gas, 100% O_2; fumarate (0·002M) in all cups except no. 1; pyruvate (0·005M) and DNP (2×10^{-4}M) added after 15 min. equilibration; fluoroacetate (0·025M) added at time indicated.)

Tissue portion	Additions	$-Q_{O_2}$ during period (min.) 20–50	50–80	80–105	105–130
1	None	2·0	1·5	1·0	1·0
2	Pyruvate	5·6	5·6	5·2	3·4
3	Pyruvate, DNP	7·5	5·8	4·9	4·4
4	Pyruvate; fluoroacetate at 15 min.	5·8	5·4	4·7	4·5
5	Pyruvate; fluoroacetate at 50 min.	5·4	5·2	4·3	3·6
6	Pyruvate, DNP; fluoroacetate at 15 min.	4·5	3·6	2·5	1·7
7	Pyruvate, DNP; fluoroacetate at 50 min.	7·1	3·2	1·7	1·2

(1947) fluoroacetate inhibits synthetic reactions and causes accumulation of acetate. This may result in a low O_2/pyruvate ratio of the same order as in the absence of the inhibitor, so that conditions may obtain in which the respiration is not appreciably altered by the addition of fluoroacetate. When added to tissue slices metabolizing pyruvate in the presence of DNP, fluoroacetate caused progressive inhibition of O_2 consumption (Table 11).

Experiments in bicarbonate saline. In bicarbonate saline in equilibrium with 5% CO_2 and 95% O_2, DNP sometimes caused a marked acceleration of the disappearance of pyruvate (Table 12), while in experiments in phosphate saline in which CO_2 was absorbed by alkali, $Q_{pyruvate}$ was not or not appreciably increased by DNP. In bicarbonate saline a large decrease of pressure was observed in the manometers containing pyruvate and DNP. This was due to the retention of CO_2, which could be recovered from the medium by addition of acid at the end of the incubation period. A smaller uptake of gas was observed when pyruvate was metabolized in the absence of DNP, while an evolution of gas occurred when glucose was the substrate. When 1 mol. of pyruvate disappears by oxidation to CO_2 and water, the liberation of one equivalent of base results in the retention of 1 mol. of CO_2 as bicarbonate. The disappearance of pyruvate by complete oxidation may therefore be formulated thus:

$$CH_3.CO.COO^- + 2{\cdot}5\ O_2 \rightarrow 2CO_2 + HCO_3^- + H_2O. \quad (1)$$

Accordingly, for every mol. of pyruvate completely oxidized, the change of pressure in the manometer should be equivalent to the uptake of 0·5 mol. of gas. In the case of incomplete oxidation of pyruvate resulting in the formation of other acidic products less CO_2 will be retained; e.g. in the reaction

$$CH_3.CO.COO^- + \tfrac{1}{2}O_2 \rightarrow CH_3COO^- + CO_2. \quad (2)$$

0·5 mol. of gas should be evolved per mol. of pyruvate disappearing.

During the complete oxidation of glucose the uptake of O_2 and the evolution of CO_2 will balance. If, however, the pyruvate formed from glucose were incompletely oxidized, an increase of pressure should be observed; e.g. in the case of the formation of acetate, 4 mol. of CO_2 would be evolved and 2 mol. of O_2 taken up, resulting in the evolution of 2 mol. of gas per mol. of glucose disappearing.

$$C_6H_{12}O_6 + O_2 + 2HCO_3^- = 2CH_3CO.COO^- + 2CO_2 + 4H_2O, \quad (3)$$

$$2CH_3.CO.COO^- + O_2 \rightarrow 2CH_3COO^- + 2CO_2, \quad (4)$$

the overall reaction being

$$C_6H_{12}O_6 + 2O_2 + 2HCO_3^- \rightarrow 2CH_3COO^- + 4CO_2 + 4H_2O. \quad (5)$$

This is illustrated in Table 12, which shows manometric pressure changes of the relative magnitude and in the direction expected from the above considerations. No attempt is made to calculate 'Δ lactic acid', i.e. to differentiate between lactic acid formed from endogenous and from added substrates, since the oxidative removal of lactic acid during incubation without added substrates would result in high 'Δ lactic acid' values. The amounts of lactic acid determined were of the same order of magnitude in the different tissue portions of each experiment, except in tissue portion 6 of Exp. 2, in which lactic acid was increased. In this case the correction for the increased CO_2 evolution due to the extra lactic acid formed reduces the observed manometric pressure change to the small value expected for the complete oxidation of glucose in the presence of DNP.

It should be pointed out that any observed pressure change (Table 12) is the resultant of the pressure changes due to the two types of reactions discussed, and may be to some degree influenced by minor side reactions. The results, nevertheless, suggest that in bicarbonate saline also, DNP causes

Table 12. *Effect of* 2:4-*dinitrophenol on gaseous exchanges of mammary tissue*

(Rat mammary gland slices incubated in bicarbonate saline (Krebs & Henseleit, 1932); gas, 5% CO_2 + 95% O_2; fumarate (0·002 M) in all cups; pyruvate, 0·005 M; glucose, 0·0025 M. Exp. 1: incubation period 100 min.; additions after 15 min. equilibration; Exp. 2: incubation period 120 min.; additions after 20 min. equilibration. Manometric pressure changes calculated as μl. CO_2.)

Exp. no.	Tissue portion	Additions	CO_2 (μl.)	Pyruvate (μl.)	Glucose (μl.)	Lactic acid (μl.)	Dry wt. (mg.)
1	1	None	−16	—	—	<10	22·8
	2	Pyruvate	−28	−86	—	22	25·1
	3	Pyruvate, 2×10^{-4} M-DNP	−103	−150	—	20	24·8
	4	Glucose	+35	—	—	27	25·3
	5	Glucose, 2×10^{-4} M-DNP	+1	—	—	37	25·5
2	1	None	−16	—	—	<40	25·7
	2	Pyruvate	−43	−215	—	100	28·1
	3	Pyruvate, 2×10^{-4} M-DNP	−146	−289	—	104	26·2
	4	Glucose	+113	—	−98	80	27·8
	5	Glucose, 2×10^{-5} M-DNP	+67	—	−114	100	25·8
	6	Glucose, 5×10^{-5} M-DNP	+73	—	−88	148	28·0

more complete oxidation of pyruvate. Further, since acetate does not accumulate in appreciable amounts during the metabolism of pyruvate

Table 13. *Respiratory quotient of mammary gland slices metabolizing pyruvate*

(Rat mammary gland slices incubated at 37° in bicarbonate saline (Krebs & Henseleit, 1932); gas, 5% CO_2 + 95% O_2; fumarate (0·005 M) and fluoride (0·005 M) in all cups; pyruvate, 0·01 M; DNP, 2×10^{-4} M; incubation period 2 hr. Method of Dickens & Šimer (1931). All added pyruvate disappeared during incubation.)

Tissue portion	Additions	$-Q_{O_2}$	Q_{CO_2}	R.Q.
1	None	2·9	3·2	1·1
2	Pyruvate	7·3	11·5	1·6
3	Pyruvate	5·6	8·4	1·5
4	Pyruvate, DNP	9·3	12·5	1·3
5	Pyruvate, DNP	8·8	11·7	1·3

(Table 6), other acidic products, e.g. fatty acids, may be formed from pyruvate in mammary tissue.

Measurements of the respiratory exchanges by the method of Dickens & Šimer (1931) showed that DNP increases the O_2 consumption of mammary gland slices metabolizing pyruvate in bicarbonate saline and at the same time decreases the R.Q. (Table 13).

DISCUSSION

Endogenous and substrate respiration of mammary tissue. In comparing the oxygen consumption and substrate utilization of a tissue which has a high endogenous respiration, the problem arises whether the increase of oxygen consumption is due to the oxidation of the added substrate, and whether and to what extent the oxidation of endogenous substrates has been reduced (cf. Potter, 1944). According to Krebs & Eggleston (1940), added pyruvate competes for oxygen with the endogenous substrates of pigeon-breast muscle and suppresses their oxidation. Since the depleted and untreated mammary gland slices show a marked difference in their endogenous Q_{O_2}, but respire at similar rates in the presence of added substrate, it can be concluded that the oxidation of added substrates depresses the high endogenous respiration of untreated mammary tissue, at least to the level observed in depleted slices. It is more difficult to assess the effect of added substrates on the low endogenous respiration of depleted tissue. This, in contrast to the endogenous respiration of untreated tissue, is not appreciably inhibited by fluoride and may to a large extent be incapable of further reduction except by disorganizing the normal metabolic reactions of the tissue. Therefore, it may be preferable to use ΔQ_{O_2}, the increase of Q_{O_2} above the endogenous level, in comparing the rates of respiration and substrate utilization of depleted tissue. At any rate the error will be small when the endogenous respiration is low and the oxygen consumption in the presence of the added substrate is high.

Krebs cycle in the mammary gland. The relationship between fumarate and malonate in the metabolism of pyruvate in mammary gland slices is similar to that originally demonstrated by Krebs & Eggleston (1940) in minced pigeon-breast muscle. Moreover, in depleted mammary tissue, small amounts of citric acid were found to accumulate during the aerobic metabolism of pyruvate. It may therefore be concluded that the citric acid cycle operates in the mammary gland. In contrast to minced pigeon-breast muscle, mammary gland slices oxidize pyruvate incompletely; the low ratio oxygen consumed/pyruvate metabolized suggests that a part of the pyruvate disappearing is not oxidized to carbon dioxide and water, but is metabolized by pathways other than the Krebs cycle.

Effect of DNP on pyruvate metabolism. The acceleration of the respiration of mammalian tissue slices by dinitrophenols was first described by Dodds & Greville (1933). As shown in the present paper, the increase in respiration caused by DNP is not accompanied by a corresponding increase in pyruvate utilization. The low ratio $\Delta Q_{O_2}/Q_{pyruvate}$, about 1 in the presence of pyruvate and fumarate, was increased to 1·5 to 2 on addition of DNP. This inhibitor has been shown to inhibit synthetic reactions in bacteria without interfering with oxidation (Clifton, 1946) and to uncouple phosphorylation from oxidation in homogenates (Loomis & Lipmann, 1948). The experimental results described are consistent with the view that the acceleration of the respiration of mammary tissue by DNP is due to the inhibition of pathways of pyruvate metabolism depending on phosphate-bond energy, i.e. synthetic reactions, with the result that a larger part of the pyruvate is oxidized via the Krebs cycle.

Pathways of pyruvate metabolism. The first evidence for the existence of two pathways of pyruvate metabolism in brain tissue, complete oxidation, and incomplete oxidation resulting in the formation of acetate, was offered by Long (1938) and Long & Peters (1939). Recently, Coxon, Liébecq & Peters (1949), working with brain homogenates, concluded that the first step in the oxidation of pyruvate is an oxidative decarboxylation, the resulting 2-carbon fragments being either stabilized as acetate, or, if oxaloacetate is available, being incorporated in the Krebs cycle. That the ratio $\Delta Q_{O_2}/Q_{pyruvate}$ is less than 1 in most experiments with mammary gland slices suggests that in this tissue complete and incomplete oxidation of pyruvate occur side by side in the presence of oxaloacetate, the observed ratio being the resultant of the ratios of the two reactions. Since acetate does not accumulate in mammary

tissue it may be presumed that the 2-carbon fragments resulting from oxidative decarboxylation of pyruvate are utilized in the synthesis of other metabolic products. If 1 mol. of pyruvate were oxidized completely and 2 mol. were utilized in the synthesis of fatty acids, e.g. of butyric acid, the overall reaction

$$3CH_3CO.COOH + 2{\cdot}5O_2 = CH_3CH_2CH_2COOH + 5CO_2 + 2H_2O \quad (6)$$

would result in an oxygen/pyruvate ratio of 0·8. This ratio would be depressed to about 0·6 if longer chain saturated fatty acid (up to C_{18}) were formed from 2-carbon units derived from pyruvate. Thus the experimental results described in this paper suggest that 1 mol. of pyruvate is oxidized for every 2 mol. of pyruvate utilized in synthetic reactions. This relationship corresponds to a Meyerhof oxidation quotient of three. Meyerhof (1925, 1942) found values of three to six for the ratio substrate disappearing/substrate oxidized for lactic acid in muscle and sugar in yeast. In the presence of DNP, however, the ratio $\Delta Q_{O_2}/Q_{pyruvate}$ approaches 2·5, and complete oxidation becomes the predominant pathway of pyruvate metabolism.

It is, therefore, most probable that in the mammary gland 2-carbon fragments derived from pyruvate are utilized in synthetic reactions which are energized by phosphate-bond energy generated during the oxidation of part of the pyruvate via the Krebs cycle (see Lipmann, 1946). Pyruvate which has long been assumed to be a key intermediate between carbohydrate breakdown and fat synthesis (see Smedley-MacLean, 1936; Bloch, 1948), has recently been shown, in isotope experiments, to be a source of fatty acid carbon *in vivo* (Anker, 1948) and in rat-liver slices *in vitro* (Brady & Gurin, 1950). Fat synthesis by mammary tissue *in vitro* from glucose or acetate has been demonstrated by Folley & French (1950) by means of R.Q. determinations. This has been confirmed by Popják, French & Folley (1951), who observed the incorporation of carbon from ^{14}C-labelled acetate into milk fatty acids of the lactating goat. In view of the importance of fat synthesis in the mammary gland (see Folley, 1949), it may be assumed that pyruvate plays a part in the synthesis of fatty acids in this tissue both as a source of metabolic energy and of fatty acid carbon.

SUMMARY

1. In order to correlate the utilization of added substrates with their effect on respiration, lactating mammary gland slices (rat, rabbit, sheep) were depleted of preformed substrates by aerobic incubation without added substrates, prior to incubation in the Warburg apparatus. By this treatment the high endogenous respiration was reduced by 50–70%; the ability of the depleted tissue to metabolize glucose or pyruvate was often impaired or lost, but was completely restored by the addition of fumarate in low concentrations.

2. Small amounts of citric acid accumulated when depleted mammary gland slices were incubated with fumarate and pyruvate. Fumarate reversed the inhibition of respiration caused by malonate. The above observations suggest that the citric acid cycle is operative in the mammary gland.

3. In phosphate saline the oxygen consumption of mammary tissue metabolizing pyruvate was increased by 50–100% by 2×10^{-4} M-2:4-dinitrophenol (DNP), without a corresponding stimulation of pyruvate utilization. DNP thus increases the ratio oxygen consumed/pyruvate metabolized. Pyruvate is metabolized with high R.Q. which is reduced to 1·2–1·3 by DNP. These findings, and manometric measurements of gaseous exchanges in bicarbonate saline, indicate that pyruvate is oxidized more completely in the presence of DNP than in its absence.

4. Although 2×10^{-4} M-DNP accelerated respiration in the presence of pyruvate, it was inhibitory in the presence of glucose. Lower concentrations of DNP (2×10^{-5} to 5×10^{-5} M) stimulated respiration when glucose was metabolized. DNP therefore appears to affect carbohydrate metabolism in two ways: (*a*) by influencing the glycolytic breakdown of glucose to pyruvate; (*b*) by influencing the metabolic fate of pyruvate.

5. It is concluded that pyruvate is metabolized by mammary tissue by two pathways, (*a*) oxidation via the Krebs cycle, and (*b*) synthetic reactions. About 2 mol. of pyruvate appear to be utilized in synthetic reactions for every mol. of pyruvate oxidized. Inhibition of synthetic reactions by DNP increases the fraction of pyruvate oxidized and thus increases the overall oxygen consumption and the oxygen/pyruvate ratio.

After this paper had been submitted for publication, a short note appeared describing the oxidation of tricarboxylic acid intermediates in lactating mammary gland cyclophorase (Moore & Nelson, 1951). This confirms the evidence presented in the present paper suggesting the operation of the Krebs cycle in the mammary gland.

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Hippuric Acid Synthesis in the Rat

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The conjugation of benzoic acid with glycine in the mammalian body which was discovered by Wöhler more than 120 years ago, has been the subject of a large number of publications. Many workers have tried to establish the source of the glycine which is made available for this conjugation without obtaining a really satisfactory answer. Lehmann (1851) suggested that the 'nitrogenous constituent of hippuric acid which we may regard as fumaramide or as glycine, is undoubtedly derived from the animal albuminous substances'. This idea was further elaborated by Wiechowski (1906), who believed that glycine or glycine precursors are formed in large amounts during the normal metabolism of proteins. Since administration of big doses of benzoate may lead to the excretion of considerable quantities of hippuric acid and a corresponding diminution of urea output, Wiechowski (1906) concluded that at least a large part of the normal catabolism of protein to urea takes place through glycine. An alternative possibility, namely that administration of benzoate stimulates the breakdown of tissue proteins and thus makes glycine available, was first put forward by Ringer (1911) and further discussed by Delprat & Whipple (1921). The observation made by various workers that the total nitrogen of the urine increased somewhat after giving large doses of benzoate was quoted as evidence for this assumption. However, other workers calculated that the glycine content of tissue proteins was not large enough to account for the amounts of hippuric acid excreted in experiments in which benzoate was fed over long periods. It was therefore suggested that the glycine was derived from other amino-acids (Magnus-Levy, 1907) or synthesized from ammonia and acetic acid (Cohn, 1893, 1905) or glyoxylic acid (Friedmann & Tachau, 1911) respectively. It was shown later that addition to the diet of glycine or of proteins rich in glycine accelerates the rate of excretion of hippuric acid in the rabbit (Griffith & Lewis, 1923) and increases markedly the output of hippuric acid in the pig (Abderhalden & Strauss, 1914; Csonka, 1924) and to a lesser extent in the dog (Quick, 1926). A similar

effect of glycine ingestion was found in man, and it was suggested that the most important factor determining the extent of hippuric acid synthesis is 'the rate at which glycine is synthesized in the body' (Quick, 1931).

More recently it was found that administration of benzoate produces in man a decrease of the content of free glycine in the plasma (Christensen, Cooper, Johnson & Lynch, 1947; de Vries & Alexander, 1948) and in the guinea pig a very marked reduction in the free glycine of liver, but not of muscle (Christensen, Streicher & Elbinger, 1948). These findings suggest that stores of free glycine in liver, kidney and plasma are primarily concerned in the conjugation. Moreover, the high isotope content of the hippuric acid obtained on simultaneous administration of ^{15}N-labelled glycine and sodium benzoate (Rittenberg & Schoenheimer, 1939; Waelsch & Rittenberg, 1941, 1942) supports such a conclusion. But the amounts of free glycine present in liver and plasma are sufficient only for the conjugation of very small amounts of benzoate, and the larger part of the glycine required under the usual conditions must ultimately be formed from other glycine stores, by synthesis or from the hydrolysis of proteins and other substances.

The question of the source of glycine used for hippuric acid formation has recently become important in the interpretation of metabolic experiments in which isotopically labelled compounds are used. Various workers (Shemin, 1946; Arnstein & Neuberger, 1949, 1951) have fed simultaneously benzoate and labelled potential precursors of glycine, isolated hippuric acid and determined its isotope content. It is clear that, if benzoate stimulates glycine synthesis or protein breakdown, the isotope content of the hippuric acid cannot be quantitatively related to normal glycine metabolism without further information.

In the present paper an attempt was made to obtain further information on this conjugation by varying the doses of labelled glycine and of benzoate, by varying the time interval between the administration of the two substances and relating the observed isotope values to these changes.

EXPERIMENTAL

Animals and diets. In all experiments but one, male and female adult albino rats of the Wistar strain supplied by Dr K. Coward, Pharmaceutical Society, were used. The body weights of the animals ranged from 220 to 460 g. For a week before the beginning of the experiment and during the experiment a low-protein diet of the following composition was fed: casein, 3; yeast, 5; salt mixture (Hubbell, Mendel & Wakeman, 1937), 4; maize starch, 80; mixture of 4 parts of cod liver oil and of 1 part of arachis oil, 8%.

Administration of glycine and sodium benzoate. Glycine labelled with ^{15}N (31·0 atom % excess) and glycine labelled with ^{14}C in the methylene group having a specific activity of 1 μc./mg. were mixed with weighed amounts of non-isotopic glycine. The glycine mixture was dissolved in water and the solution made up to a suitable volume. Sodium benzoate, A.R., was dissolved in water to a final concentration of 5 or 10% (w/v) depending on the dose. In one set of experiments the dose of glycine was kept low and constant while the dose of benzoate was varied within wide limits. In another experiment the dose of glycine was varied while the dose of benzoate was kept constant and in a third type of experiment doses of both glycine and benzoate were kept constant and the time interval between administration of the former and of the latter was varied. Both glycine and benzoate were, except in one experiment (p. 157), administered intraperitoneally by injection.

Collection of hippuric acid and determination of isotope content. The rats were kept during the experiments in metabolism cages and the urine was collected for 24 hr. after injection of the benzoate. The hippuric acid was isolated as described (Arnstein & Neuberger, 1951) and recrystallized from water to constant melting point. In several experiments the specific radioactivity was determined after successive recrystallizations from water. No significant change was found after one recrystallization.

The excess ^{15}N in the hippuric acid was measured in the mass spectrometer (Sprinson & Rittenberg, 1949). The specific radioactivities were determined with a bell-shaped helium-filled Geiger-Müller counter having a thin mica window. Background count was 11–13 counts/min. The measurements were made on solid samples spread evenly on disks of identical geometry and having 1 sq.cm. area. Each sample contained at least 25 mg./sq.cm. and the number of counts is therefore linearly proportional to activity. No correction for self-absorption was made. In one experiment some of the glycine used for the injections was converted to hippuric acid *in vitro* by the Schotten-Baumann method and the specific activity of the latter determined. This produced a satisfactory check on the calculation of dilution factors which were obtained by comparison of the specific activities of the injected glycine and of the isolated hippuric acid. Results are expressed as counts/min.

Nitrogen balance. In one experiment a group of seven female albino rats of body weight ranging from 245 to 260 g. was put for 2 weeks on the low-protein diet fed *ad lib.* Food consumption was measured accurately and the total N of the urine was determined. The rats were kept in metabolism cages and the urine collected over periods of 24 hr. The cages, wire screens and funnels were washed down with water, the combined washings and urine were filtered and the volume made up to 100 ml. N was determined by the micro-Kjeldahl method on 1 ml. samples. The rats were then given subcutaneously varying doses of sodium benzoate together with a constant amount of ^{15}N-labelled glycine. Daily food consumption and urinary N excretion were measured for 1 week after injection. Hippuric acid was isolated and its ^{15}N content determined.

RESULTS

Nitrogen balance and hippuric acid formation

In the first experiment, the details of which are given in Table 1, the effect of benzoate, administered subcutaneously, on nitrogen balance was determined. During the first period, before the injection of the benzoate, the average daily food

intake amounted to 14·0 g./rat and the average urinary N excretion was 78 mg./rat/day. After injection of the benzoate, food intake fell sharply in all animals. It dropped about 8 g./rat/day for the first 3 days in the four rats which had received 0·10 or 0·30 mmol. benzoate/100 g. and reached the normal level again 6 days after injection. With doses of 0·5 or 0·75 mmol. benzoate/100 g. food intake decreased during the first 3 or 4 days to about 5 and 3 g./rat/day respectively and increased to about 10 g./day at the end of 1 week. The daily urinary N did not increase in the two rats which had received the lowest dose of benzoate, but rose to about 100 mg. the day after the injection in the two rats given 0·30 mmol. benzoate/100 g. With the three rats which received the larger doses of benzoate the urinary N increased the day after the injection to between 120 and 150 mg./day. Normal levels of N excretion were again obtained 5 days after the injection. All animals showed a loss of weight. Injection of benzoate thus produced in all rats a marked negative N balance and the loss of nitrogen from the body was greater than could be accounted for by the glycine required for the conjugation with benzoate.

Griffith (1929) has shown that continuous feeding of a diet containing 35 % casein and varying amounts of benzoate to rats produces loss of weight and reduction of food intake. The effects found in our experiments were apparently more severe than those observed by Griffith; this may be due to the very low protein content of our diet. However, the subcutaneous administration of benzoate used in this first experiment produced lesions on the site of the injection and thus local inflammatory reactions may have enhanced the systemic toxic effect of benzoate. In subsequent experiments we therefore used the intraperitoneal route.

The effect of the dose of benzoate on the isotope content of hippuric acid

Simultaneous administration of benzoate and glycine. Increase of the dose of the benzoate reduced markedly the ^{15}N content of hippuric acid as shown in Table 1, which also demonstrates the uniformity of response of different animals to identical doses of glycine and benzoate. Thus the differences in isotope content of hippuric acid excreted by animals which had received similar treatment rarely exceeded 15 %. In a second experiment a mixture of ^{14}C- and ^{15}N-labelled glycine was given, the lowest dose of benzoate was reduced to 0·05 mmol./100 g. and the substances were injected intraperitoneally. In this and following experiments the urines of animals receiving identical doses of benzoate/unit body weight were combined. A comparison of the results of the two experiments (Tables 1 and 2) shows that the dilution factors and even more the dilution coefficients, which take the dose of glycine/unit body weight into account, were very similar for identical doses of benzoate. Thus the dilution coefficient was about four to five times greater with the highest doses of benzoate than with the lowest dose investigated. Reduction of the dose of benzoate from 0·1 to 0·05 mmol./100 g. produced a marked change in isotope content and it is likely, therefore, that a further decrease of the amount of benzoate administered would have further increased the isotope content of the hippuric acid. All the results obtained with simultaneous administration of variable amounts of benzoate and more or less constant amounts of glycine have been collected in Fig. 1. The relationship between benzoate concentration and isotope content of hippuric acid appears to be neither strictly linear nor exponential and extrapolation to zero benzoate concentration would therefore be hazardous. It would seem, however, that the corrected dilution coefficient approaches a value of about 0·1 mmol./100 g. body weight or less, as the amount of benzoate administered becomes very small.

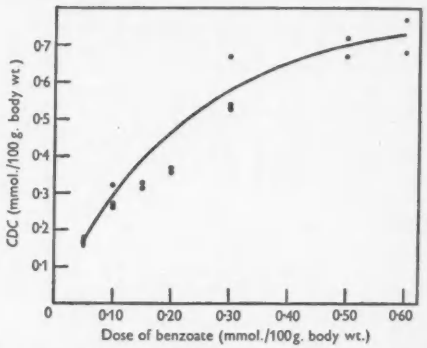

Fig. 1. The effect of dose of benzoate on the isotope content of hippuric acid (expressed as corrected dilution coefficient (C.D.C.), see Table 1) on simultaneous administration of labelled glycine and sodium benzoate. Some of the experimental points are taken from Tables 1 and 2.

Effect of administration of benzoate at various times after that of glycine. When differing doses of benzoate were given to rats which had received 3 hr. earlier identical doses of glycine/unit body weight, the isotope content of hippuric acid still varied with the benzoate concentration (Table 3), but not quite as much as in the earlier experiments (Tables 1 and 2) in which glycine and benzoate were given simultaneously. When, however, the benzoate was injected 6 hr. after glycine had been given, the isotope content of the hippuric acid was independent of the dose of benzoate (Table 3). A similar result was obtained, when the interval between the administration of the two substances was increased to 24 hr.

In this experiment 0·044 mmol. glycine (containing 31·0 atom % excess ^{15}N)/100 g. body weight was injected into twelve rats. Amounts of benzoate similar to those used in the earlier experiments (0·1–0·6 mmol./100 g. body weight) were injected 24 hr. later and the hippuric acid isolated. The mean ^{15}N contents of the hippuric acid samples were 0·118, 0·120, 0·142, 0·122 and 0·128 atom % excess. Thus there was no evidence for a variation of ^{15}N content with the amount of benzoate administered.

In another experiment (Table 4) the interval was increased to 67·5 hr. The individual variations appear to be somewhat greater than in the earlier experiments, but again there was no evidence for a diminution of isotope content with increasing amounts of benzoate administered.

The ratio of ^{14}C and ^{15}N content in the isolated hippuric acid compared with that of the injected glycine. In many of the experiments glycine labelled with ^{14}C and ^{15}N was used and both isotopes were determined in the hippuric acid. The probable error of the 'isotope ratio', i.e. the quotient of the dilution factor for ^{14}C over that for ^{15}N, is a function of the mean errors of four separate estimations. The isotope content of the administered glycine was estimated with an

Table 1. *^{15}N contents of hippuric acid samples excreted by rats which had been injected subcutaneously with ^{15}N-labelled glycine (31·0 atom % excess) and varying doses of sodium benzoate at the same time*

(The animals were female albino rats of body weight between 225 and 260 g.; they received 10 mg. isotopic glycine/100 g. body weight. The corrected dilution factor (C.D.F.) is defined as $C_0/C - 1$, where C_0 is the isotope content of administered glycine and C the isotope content of the excreted hippuric acid. The corrected dilution coefficient (C.D.C.) is defined as $(C_0/C - 1)\,Q$, where Q is the administered dose of glycine in mmol./100 g. body weight. This form of representing results is considered in the Discussion, p. 159.)

Rat no.	Dose of benzoate (mmol./100 g. body wt.)	^{15}N content of hippuric acid (atom % excess)	C.D.F.	C.D.C. (mmol./100 g. body wt.)
101	0·1	4·380	6·1	0·32
102	0·1	5·035	5·1	0·27
103	0·3	2·612	10·8	0·57
104	0·3	2·830	10·0	0·53
105	0·5	2·280	12·6	0·67
106	0·5	2·115	13·6	0·72
107	0·75	1·409	20·7	1·10

Table 2. *Isotope contents of hippuric acid samples excreted by rats which had been injected intraperitoneally with doubly labelled glycine and sodium benzoate simultaneously*

(The glycine contained 15·5 atom % excess ^{15}N and gave 866·5 counts/min. Each animal received 0·033 mmol. glycine/100 g. body weight. Where small doses of benzoate were given, the urines of rats which had received identical amounts of benzoate/unit body weight were combined as indicated in the table. For definition of corrected dilution factor (C.D.F.) and corrected dilution coefficient (C.D.C.) see Table 1. Isotope ratio is the quotient of the ratio of the radioactivity of the injected glycine to that of the excreted bound glycine (both expressed as counts/min.) by the ratio of the ^{15}N content (atom % excess) of the injected glycine to that of the excreted hippuric acid. The radioactivity of the excreted glycine was obtained by multiplying the observed radioactivity of the hippuric acid by the ratio of molecular weight of hippuric acid to that of glycine.)

Rats no.	Dose of benzoate (mmol./100 g. body wt.)	Hippuric acid: *^{14}C content (counts/min.)	Hippuric acid: ^{15}N content (atom % excess)	C.D.F. from results with: ^{14}C	C.D.F. from results with: ^{15}N	C.D.C. (mmol./100 g. body weight) from results with: ^{14}C	C.D.C. (mmol./100 g. body weight) from results with: ^{15}N	Isotope ratio
1, 2, 3	0·05	138	2·467	5·2	5·3	0·172	0·175	0·98
4, 5, 6	0·10	95·6	1·737	8·1	7·9	0·267	0·260	1·03
7, 8	0·15	79·8	1·476	9·9	9·5	0·327	0·314	1·04
9, 10	0·20	73·2	1·278	10·8	11·1	0·356	0·366	0·97
11	0·30	52·2	0·890	15·6	16·4	0·515	0·541	0·95
12	0·60	35·5	0·716	23·4	20·7	0·772	0·683	1·13

* Calculated for glycine.

Table 3. *Effect of changes in the dose of benzoate on the isotope contents of hippuric acid excreted by rats which had received doubly labelled glycine and, 3 or 6 hr. later, sodium benzoate*

(Each animal received 0·044 mmol. glycine/100 g. body weight; the glycine contained 15·62 atom % excess ^{15}N and had radioactivity equivalent to 7460 counts/min. of hippuric acid. For definition of C.D.F. and C.D.C. see Table 1 and for definition of isotope ratio see Table 2.)

Rats no.	Dose of benzoate (mmol./100 g. body wt.)	Hippuric acid: ^{14}C content (counts/min.)	Hippuric acid: ^{15}N content (atom % excess)	C.D.F. from results with: ^{14}C	C.D.F. from results with: ^{15}N	C.D.C. (mmol./100 g. body weight) from results with: ^{14}C	C.D.C. (mmol./100 g. body weight) from results with: ^{15}N	Isotope ratio
Benzoate given 3 hr. after glycine								
37, 38, 39	0·05	163·7	0·357	44·6	42·7	1·96	1·88	1·04
40, 41, 42	0·10	190·3	0·363	38·2	41·2	1·68	1·81	0·95
43, 44	0·15	130·5	0·305	56·2	50·2	2·47	2·21	1·11
45, 46	0·20	115·8	0·289	63·4	53·6	2·81	2·33	1·19
47	0·30	105·9	0·253	69·4	60·7	3·05	2·67	1·14
48	0·60	72·6	0·189	101·7	81·6	4·47	3·59	1·24
Benzoate given 6 hr. after glycine								
49, 50, 51	0·05	—	0·153	—	101	—	4·44	—
52, 53, 54	0·10	63·3	0·158	117	97	5·15	4·27	1·20
55, 56	0·15	60·6	0·149	123	104	5·41	4·58	1·18
57, 58	0·20	62·1	0·151	119	102	5·24	4·49	1·16
59	0·30	56·1	0·145	132	107	5·81	4·71	1·23
60	0·60	56·9	0·140	130	111	5·72	4·88	1·17

— means value not available.

Table 4. *Effect of changes of the dose of benzoate on the isotope content of hippuric acid excreted by rats which had received doubly labelled glycine and 67·5 hr. later sodium benzoate*

(Each animal received 0·044 mmol. glycine/100 g. body weight. The glycine contained 25·2 atom % excess ^{15}N and had radioactivity equivalent to 161,000 counts/min. counted as hippuric acid. The samples of hippuric acid excreted contained between 0·045 and 0·063 atom % excess ^{15}N with a mean value of 0·053 atom % excess and corresponding C.D.F. value of 475. For definition of C.D.F. and C.D.C. see Table 1.)

Rats no.	Dose of benzoate (mmol./100 g. body wt.)	Radioactivity of hippuric acid (counts/min.)*	C.D.F.	C.D.C. (mmol./100 g. body wt.)
31, 32, 33	0·10	238·5	675	29·7
29, 30	0·15	229·5	700	30·8
27, 28	0·20	354·0	454	20·0
26	0·30	269·0	597	26·3
25	0·60	344·0	466	20·5

* Calculated as glycine.

Table 5. *Effect of the dose of glycine on the isotope content of hippuric acid excreted by rats after simultaneous administration of ^{14}C-labelled glycine and sodium benzoate*

(Fractions of approx. 0·1 ml. of solution of labelled glycine (10·2 mg.; activity: 663,100 counts/min.) were weighed out; these were mixed with appropriate amounts of an aqueous solution of non-labelled glycine or of water. Fractions of the diluted solutions were then injected intraperitoneally into four groups of rats (three in each group) so as to give the doses of total glycine/100 g. body weight stated in the table. A solution of sodium benzoate (0·05 mmol./100 g. body weight) was injected at the same time. For definitions of corrected dilution factor (C.D.F.) and corrected dilution coefficient (C.D.C.) see Table 1.)

	Glycine given				
Rats no.	Amount (μmol./100 g. body wt.)	Activity (counts/min.)	Activity of hippuric acid (counts/min.)	C.D.F.	C.D.C. (mmol./100 g. body wt.)
76, 77, 78	44·4	1,041	87·8	3·97	0·166
73, 74, 75	11·16	3,585	99·6	14·08	0·157
70, 71, 72	2·84	14,705	108·4	56·0	0·159
67, 68, 69	0·061	663,100	96·7	2873	0·178

Table 6. *Effect of change in the time interval between the administration of glycine and that of benzoate on the isotope content of hippuric acid excreted by rats*

('Black and white' rats weighing about 250 g. were each given intraperitoneally 10 μc. $CH_3{}^{14}COONa$ and 0·4 mmol. glycine (31·0 atom % excess ^{15}N)/100 g. body weight. After intervals given in the table the animals received sodium benzoate (0·42 mmol./100 g. body weight) and ethyl DL-α-amino-phenylbutyrate (0·3 mmol./100 g. body weight). For definition of corrected dilution factor (C.D.F.) and corrected dilution coefficient (C.D.C.) see Table 1. The activities of the acetamido acid are reported by Arnstein & Neuberger, 1951.)

Rat no.	Time between injection of glycine and of benzoate (hr.)	^{15}N content of hippuric acid (atom % excess)	C.D.F.	C.D.C. (mmol./100 g. body wt.)
201	0	10·59	1·93	0·77
202	0	7·29	3·12	1·25
203	3	3·35	8·22	3·29
204	3	3·43	8·05	3·22
205	7	1·88	15·5	6·2
207	12	1·11	27·0	10·8
208	12	1·26	23·5	9·4
211	24	0·84	35·9	14·4

accuracy of ±1·5–2·5 %, but for the hippuric acid the error is somewhat larger, especially for samples of low isotope content, and may be as high as ±6·0 %. It is believed that the values of the isotope ratio in Table 2 do not differ significantly from 1·0, but some of the isotope ratios in Table 3 appeared to be somewhat outside the probable limits of error. Some of the glycine used for the injection was therefore converted to hippuric acid *in vitro* and its ^{15}N and ^{14}C content estimated. Similarly, most of the hippuric acid samples were recrystallized, counted again and the ^{15}N analysis repeated. The results differed only slightly from those obtained initially. The values in Table 3 are almost consistently above 1·0 with a mean of about 1·15. This may be due to an undetected analytical error or it may indicate

a breakdown of part of the glycine followed by a synthesis in which the nitrogen is re-incorporated to a greater extent than the methylene carbon atom.

The effect of the amount of glycine administered on the isotope content of the hippuric acid. In one experiment the amount of glycine administered was varied between 44·4 and 0·061 mmol./100 g. body weight, whilst the dose of benzoate was kept constant at 0·05 mmol. The results (Table 5) show that, whilst the dilution factor varies within wide limits, the dilution coefficient remains remarkably constant. However, with very high doses of glycine, such as those employed in another experiment (Table 6), the corrected dilution coefficient seems to be about 30% higher than was found with small amounts of glycine using similar intervals and benzoate doses (Tables 1–3).

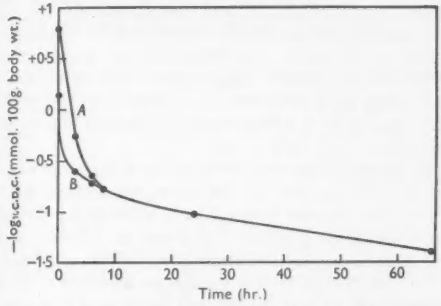

Fig. 2. The effect of the length of the interval between administration of labelled glycine and that of benzoate to rats on the isotope content of excreted hippuric acid. Curve *A* refers to low benzoate doses (0·05 mmol./100 g. body weight), whilst curve *B* refers to high benzoate doses (0·5 to 0·6 mmol./100 g. body weight). C.D.C. is defined in Table 1.

The decrease in isotope content with time of the glycine available for hippuric acid synthesis. If the doses of benzoate and of glycine administered are kept constant and the interval between injection of glycine and of benzoate is varied, the isotope content of the hippuric acid may be expected to yield information about the turnover rate of that 'glycine fraction' which is used in hippuric acid formation. During an investigation on the biosynthesis of glycine from acetate (Arnstein & Neuberger, 1949, 1951) ^{15}N-labelled glycine (0·4 mmol./100 g. body weight) was injected into a group of rats, followed after differing intervals by administration of benzoate. The marked diminution in isotope content which occurred in the first 7 hr. was followed by a slower decrease in the succeeding 17 hr. (Table 6). A similar curve was obtained by plotting against time the values found in the earlier experiments (Tables 1–4) with high benzoate concentrations (Fig. 2, curve *B*). If experiments with low benzoate concentrations (0·05 mmol./100 g. body wt.) are compared, the difference between the sharp fall in the first few hours after administration of glycine and the relatively slow decrease occurring later becomes even more marked (Fig. 2, curve *A*). Thus, the isotope content of the glycine used for hippuric acid formation diminished to about one twenty-fifth during the first 6 hr., whilst at the end of the following 60 hr. the further decrease was only to about one-fifth of the 6 hr. value.

DISCUSSION

The significance of dilution factors

The quantitative interpretation of experiments in which labelled compounds are fed to an animal presents difficulties which are largely absent in work on isolated tissue homogenates or enzyme systems. The complexity of the various catabolic and anabolic reactions and our lack of knowledge of the rates of absorption and penetration into cells of the substances under consideration make it necessary to use as a first approach a highly simplified model which is admittedly inadequate, but which can be made more elaborate in the light of further observations.

If an isotopically labelled substance A such as glycine of label content C_Q is fed in an amount Q (in mmol./100 g. body weight) and A, or a metabolic derivative thereof, is isolated with a label content C, then the ratio C_Q/C is called the dilution factor (Bloch & Rittenberg, 1945). In order to give a physical significance to this factor we assume that the injected or fed glycine mixes very quickly after administration with a fraction or the whole of the free glycine present in the body. This 'first glycine pool' is assumed to be homogeneous with respect to glycine and is considered to be an isolated system, i.e. the rate of diffusion of glycine from one part of the pool to another is assumed to be infinitely fast compared with any other reaction glycine may undergo, whilst diffusion into any other part of the body is assumed to be infinitely slow. It is also postulated that the labelled glycine is quantitatively transferred to this pool and that the rate of this transfer is fast; in other words, absorption is supposed to be quantitative and almost instantaneous. Under such conditions the following equation must hold:

$$\frac{x+Q}{Q}=\frac{C_Q}{C}, \qquad (1)$$

where x is the amount of glycine (in mmol./100 g.) present in the 'first glycine pool'. From this we get

$$\frac{x}{Q}=\frac{C_Q}{C}-1. \qquad (2)$$

In some of the present experiments the dilution factors are small and the use of $C_Q/C-1$, which is named the corrected dilution factor (C.D.F.), is preferable (see Bloch & Rittenberg, 1945). The dilution factor gives only incomplete information about metabolic interrelationships, since the dose of the administered, labelled material and the weight of the animal are not taken into account. Bloch & Rittenberg (1945) have therefore used the product of the dilution factor and of the dose (in mmol./100 g. rat/day) to calculate acetate formation in the rat. We shall use x as defined by equation (1) for a similar purpose, and call it the corrected dilution

coefficient (C.D.C.). The labelled glycine in our experiments was given over an extremely short time and C.D.C. has therefore the dimensions mmol./100 g. of rat.

In this investigation the glycine in the pool after mixing was not isolated as such, but the benzoate which was given was supposed to combine specifically with this free-glycine fraction. For this sampling method to be valid three further conditions have to be fulfilled. The rate of absorption of the benzoate has to be fast relative to the turnover rate of the glycine pool. The conjugation of glycine with the benzoate has to be slow compared with the rate of diffusion of glycine within the pool, i.e. the mixing of the glycine within the 'first pool' has to be complete, before hippuric acid formation takes place. But, on the other hand, this conjugation has to be fast compared with any metabolic reaction of glycine or with the diffusion of glycine to any part of the body outside the pool. If these conditions are met, the isotope content of the excreted hippuric acid is a direct measure of C for glycine.

This highly simplified picture yields convenient formulae for the presentation of results of metabolic experiments in which isotopes are used. The assumptions made are, at the best, crude approximations, and it will have to be considered in the light of other evidence how far such an expression as C.D.C. represents an identifiable fraction of the body glycine.

The effect of dose of benzoate on the isotope content of hippuric acid

When the interval between the injection of the labelled glycine and that of benzoate was 6 hr. or more, the isotope content of the hippuric acid was found to be independent of the dose of benzoate. This finding strongly suggests that the administration of benzoate to the rat does not directly increase the rate of synthesis of glycine. If such a stimulation of glycine synthesis were to occur, it should be proportional to the amount of benzoate given and thus lead to a progressive diminution of isotope content of the hippurate with increasing dose of benzoate. If glycine synthesis from non-nitrogenous precursors were to take place, we should also expect a marked change in the ratio of ^{14}C to ^{15}N in the excreted hippuric acid as compared with that of the administered glycine. In fact, the 'isotope ratio' never differed greatly from 1·0, and such increases of 'isotope ratio' as did occur were not correlated with the dose of benzoate. The possibility that administration of benzoate produces an increase in the rate of liberation of glycine from proteins cannot be completely excluded. The relatively small increase in nitrogen excretion on giving benzoate observed by ourselves and other workers suggests that proteolysis is not markedly raised. Such increases of urinary nitrogen excretion as were observed occurred mainly 1–2 days after the administration of benzoate and are probably associated with the regeneration of the free glycine level in the tissues. Our results show that, if benzoate brings about increased protein breakdown, the additional glycine thus made available must have the same isotope content 6 hr. after the injection of the labelled glycine as the free glycine which is primarily utilized for conjugation of benzoate. Preliminary experiments in this laboratory indicate that the total protein nitrogen of rat liver is not significantly reduced by injection of 0·3 mmol. benzoate/100 g. of rat, and it is therefore believed that proteolysis is not markedly increased during hippuric acid formation.

These conclusions apply only to the rat. In man, evidence has been obtained (Gray & Neuberger, 1950) which suggests that administration of benzoate may lead either to an increased synthesis of glycine or to a mobilization of the glycine bound in proteins.

When benzoate was administered, together with the labelled glycine or 3 hr. later, the isotope content of the hippuric acid fell with increasing doses of benzoate. This may be explained as follows: the investigations of various workers (Griffith & Lewis, 1923; Csonka, 1924; Bray, Thorpe & White, 1951) suggest that in rabbit and man the rate of hippuric acid synthesis is more or less independent of the dose of benzoate, and this presumably also applies to the rat. An increase of dose of benzoate is therefore equivalent to an extension of the time interval during which conjugation takes place. During the first 4–6 hr. after injection of labelled glycine the isotope content of the free-glycine fraction which is utilized for hippurate formation falls very sharply (Fig. 2), whilst later the rate of decrease with time is relatively small. It is therefore easy to understand why an increase of dose of benzoate produces a decrease in isotope content during the first few hours after glycine injection, but not later.

The source of glycine used for hippuric acid formation

For a more quantitative interpretation of the results we shall have to consider the amounts of free glycine and of precursors of free glycine present in various tissues of the rat, the rates of absorption and of transfer of glycine from one compartment of the body to another and the rates of some relevant metabolic reactions. The data on the amounts of free glycine in tissues (Todd & Talman, 1949; Awapara, 1949; Krueger, 1950) are not in very good agreement, but the following approximate figures (expressed in mg./100 g. body weight) may be assigned to the various tissues of the rat: blood, 0·25; kidney, 1·2–1·4; intestine, 3·0; liver, 1·2–2·9; muscle, 25–30. It is likely that the total free glycine of the internal organs is about 5–7 mg./100 g. body

weight, whilst the total free glycine in the whole rat is about 30–45 mg. When labelled glycine is injected, it will be taken up very quickly by the cells of the liver, kidney and intestine. This follows from the isotope experiments of Hultin (1950) on chicks, and those of Borsook, Deasy, Haagen-Smit, Keighley & Lowy (1950) on mice. It appears that muscle cells are not very permeable to glycine (Van Slyke & Meyer, 1913; Luck, 1928; Friedberg & Greenberg, 1947; Handler, Kamin & Harris, 1949; Kamin & Handler, 1951), and it is probable that equilibration of the labelled glycine with the intracellular glycine of muscle will be relatively slow.

In experiments in which labelled glycine and a very small amount of benzoate were injected to-

rate of conjugation of benzoate. This conclusion is supported by the observations that the isotope content of hippuric acid is not markedly affected by the dose of glycine in which a constant amount of isotope is administered, at least within wide limits (Table 5). The figure for the 'first glycine pool' is much lower than the total free glycine of the body and this finding is compatible with the assumption that with small amounts of benzoate conjugation is complete before any extensive mixing with the free intracellular glycine of tissues other than those of the internal organs has taken place.

The interpretation offered here postulates that the isotope content of the hippuric acid is a measure of the mean isotope content of the free glycine of the

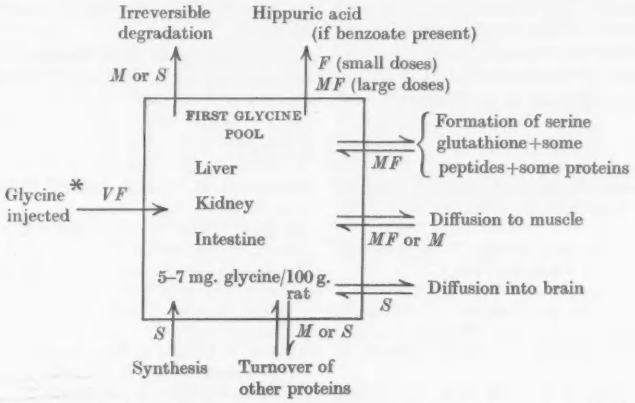

Fig. 3. Diagrammatic representation of possible fate of glycine. Rates of reactions indicated as follows in decreasing order: *VF*, very fast; *F*, fast; *MF*, moderately fast; *M*, moderate (come to equilibrium within 4–5 hr. after injection); *S*, slow (not near equilibrium after 4–5 hr.).

gether (Table 2), a value for C.D.C. or 'first glycine pool' of about 0·17 mmol./100 g. or 10 mg./100 g. of rat was obtained. It is possible that a lower value might have been found, if it had been possible to reduce further the dose of benzoate. In any case the figure of 10 mg. is not very much higher than the value of the estimated free glycine content of the internal organs which is approx. 5–7 mg. With any finite amount of benzoate, conjugation will require a period of time during which the labelled glycine will be diluted owing to incorporation into proteins, the occurrence of other reversible and irreversible reactions and also due to some penetration into cells of tissues other than those of internal organs. It is thus not surprising that even the lowest value for C.D.C. is higher than the amount of free glycine in internal organs. It is suggested that the rate of mixing of labelled glycine with the glycine of liver and that of other organs with similar permeability characteristics is relatively fast compared with the liver over a time interval, the length of which is a function of the dose of benzoate. The time curve (Fig. 2) is thus a rough representation of the changes of the isotope content of free glycine of rat liver after intraperitoneal injection of labelled glycine. This curve indicates that during the first 4–5 hr. after injection the isotope content falls sharply. This rapid decrease must be caused by relatively fast reactions such as formation of glutathione and serine, incorporation of glycine into proteins with high turnover rates and diffusion of glycine into muscle and possibly brain cells. It would also appear that the 'half-life' of the free liver glycine is extremely short. After about 6 hr. the total free glycine in the body, the serine and the glycine bound in glutathione and in some protein or peptide fractions are likely to be in approximate equilibrium with respect to their isotope contents. The much slower rate of dilution observed in the later part of the curve (Fig. 2) will represent mainly the re-

latively low turnover rates of the bulk of the proteins of the internal organs and of muscle and other proteins of the body. Diminution of isotope content will also occur owing to synthesis of glycine which will affect both parts of the curve.

Whilst accurate information about the rates of relevant reactions is lacking, the present results are compatible with the interpretations discussed above and which are diagrammatically represented in Fig. 3.

SUMMARY

1. Hippuric acid formation by the rat has been studied by injecting graded doses of sodium benzoate and ^{15}N- and [α-^{14}C]-labelled glycine at different time intervals and isolating the excreted hippuric acid. With doubly labelled glycine no marked change in the $^{15}N/^{14}C$ ratio was found.

2. With simultaneous administration of benzoate and labelled glycine, increase of the benzoate dose diminished markedly the isotope content of the excreted hippuric acid.

3. Injection of varying doses of benzoate 3 hr. after the glycine resulted in only slight variations of the isotope content of the hippuric acid; with time intervals of 6 hr. or longer there was no variation.

4. Within wide limits the quantity of glycine in which a given amount of isotope was administered had little or no effect on the isotope content of the hippuric acid.

5. It is concluded that the amount of glycine immediately available for conjugation with small doses of benzoate is about 10 mg./100 g. body weight. This 'first glycine pool' is in relatively slow equilibrium with a larger 'pool' of glycine and glycine precursors, which participate in hippuric acid formation only when larger doses of benzoate are given.

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A New Method for Degradation of *n*-Carboxylic Acids

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In the course of studies on the mechanism of the biosynthesis of fatty acids, carried out with the aid of isotopically labelled precursors, it became desirable to degrade various long and short chain acids in order to determine the position of the labels in the carbon chains. The methods described in the literature were not considered satisfactory for the scale of work envisaged, although the Barbier-Wieland method (Lane & Wallis, 1941) has been used successfully by Stadtman & Barker (1949) to degrade the hexanoic acid synthesized by *Clostridium kluyveri*. The conversion of the silver salt of a fatty acid under the action of bromine into an alkyl bromide with loss of carbon dioxide (Hunsdiecker & Hunsdiecker, 1942; Oldham, 1950) has also been considered in an alternative scheme. The alkyl bromide might be further degraded to a lower carboxylic acid as shown in Fig. 1.

However, a more than century-old observation was brought to our notice by Dr J. W. Cornforth, which suggested a simpler method of degradation involving simultaneous removal of two carbon atoms as acetic acid from the carboxyl end of the fatty acid. Varrentrap (1840) showed that fusion of oleic acid with solid potassium hydroxide led to the formation of palmitic acid in very good yield, the other main product being acetic acid. Apparently, under the influence of molten potassium hydroxide, the double bond migrated from the 9:10 to the 2:3 ($\alpha\beta$) position before fission of the molecule took place. This observation was confirmed by Edmed (1898), and a few years later a similar reaction was shown to occur when octadec-2-enoic acid was fused with solid potassium hydroxide (Ponzio, 1905). Similarly, decanoic acid has been prepared by the fusion of dodec-2-enoic acid with potassium hydroxide (Kao & Ma, 1931). We have found also that under similar conditions hex-2-enoic acid gave butyric and acetic acid in 80–90 % yield. The method of fatty acid degradation to be described is based on this type of reaction. A preliminary account of some of this work has already been published (Hunter & Popják, 1951).

METHODS AND DISCUSSION

The method of degradation developed involves the following steps: bromination of the carboxylic acid chloride (I), conversion of the brominated product to an ester (II) by a method similar to that used by Blaise & Picard (1912), and dehydrobromination of the ester. The resulting unsaturated ester (III) is then saponified and the free acid fused with potassium hydroxide at 300–340° to yield acetic acid and another saturated carboxylic acid (IV) two carbon atoms shorter than the original acid. These steps may be represented by

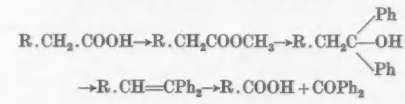

Fig. 1. The Barbier-Wieland and silver salt methods of fatty acid degradation.

$$\underset{}{R.CH_2.CH_2COOH} \rightarrow \underset{}{R.CH_2CH_2COCl} \rightarrow \underset{(I)}{R.CH_2CHBrCOCl}$$

$$\rightarrow \underset{(II)}{R.CH_2CHBr.COOR'} \rightarrow \underset{(III)}{R.CH{=}CH.COOR'}$$

$$\rightarrow R.CH{=}CH.COOH \rightarrow \underset{(IV)}{R.COOH} + CH_3COOH.$$

Model experiments were carried out on hexanoic acid and extended to other normal straight-chain acids. It was hoped that it might be possible to

effect dehydrobromination and fission in one stage by fusion of α-bromohexanoic acid with solid potassium hydroxide. However, the process gave rise to pentanoic (valeric) acid and to only small quantities of butyric and acetic acids. Attempted direct dehydrobromination of the free α-bromo-acid with collidine or quinoline likewise failed to produce the desired result; only the formation of the corresponding lactide ester was observed, which might have been formed by intermolecular elimination of hydrogen bromide. The dehydrobromination of ethyl α-bromohexanoate had, however, been carried out successfully with quinoline (Fichter & Pfister, 1904) or with diethylaniline (Blaise & Lüttringer, 1905), and attention was therefore turned towards the esters. It was found that methyl hex-2-enoate, prepared from an authentic sample of hex-2-enoic acid, could be degraded directly to butyric and acetic acids by fusion with potassium hydroxide in almost as high a yield as the free acid. It was surprising to find, however, that, in contrast with ethyl α-bromohexanoate, the methyl ester could not be dehydrobrominated under any of the variety of conditions tried, only varying amounts of the lactide ester of α-hydroxyhexanoic acid being obtained. This difference in reactivity of the methyl and ethyl esters suggested a trial of other more complex esters in order to improve the yield from the degradative process.

It seemed likely that a major side reaction causing a reduced yield during dehydrobrominations might occur as a result of nucleophilic attack of freshly liberated bromide ions upon the α-carbon atom

Table 1. *Yields of esters of α-bromohexanoic acid and of hexenoic acid obtained with straight-chain alcohols*

Ester	Yield of α-bromohexanoate (%)	Yield of hexenoate from hexanoic acid (%)
Methyl	68	0
Ethyl	76	11
n-Propyl	81	19
n-Octyl	67	23

of the alcohol participating in the ester grouping. The isolation of some lactide ester in the course of attempted dehydrobromination of the methyl ester of α-bromohexanoic acid provided some evidence for this hypothesis. Accordingly, it became desirable to synthesize esters which would hinder the approach of bromide ions towards the ester grouping. Pursuing these ideas, it was found that the dehydrobromination of *n*-propyl α-bromohexanoate gave a considerably better yield of the ester of the ethenoid acid than the same reaction on the ethyl ester. However, further lengthening of the carbon chain had little more effect, the dehydrobromination of *n*-octyl α-bromohexanoate proceeding in only slightly better yield.

Next, the effect on the reactions of the substitution of methyl groups in the α-position of the alcohol was investigated. *iso*Propyl α-bromohexanoate again proved superior to its ethyl analogue, but further substitution was disappointing. The *tert.*-butyl ester of α-bromohexanoic acid formed in only moderate yield from *tert.*-butanol and the dehydrobromination of the product also proceeded unsatisfactorily, presumably due to the elimination of *iso*butene from the ester at the elevated temperature (approx. 180°) necessary for the reaction.

If the yield-reducing process in the dehydrobromination reaction was in fact brought about by the attack of bromide ions, there was a formal analogy here to the extensively studied interaction of nucleophilic reagents with alkyl halides. Whitmore & Fleming (1933) have shown that β-substituted alkyl halides, especially neopentyl halides, are very inert towards the ordinary nucleophilic reagents. These facts have been interpreted as arising from the large steric hindrance to the approach of attacking ions exerted by these halides (Dostrovsky, Hughes & Ingold, 1946, 1948).

The best results were indeed achieved by substitution of methyl groups in the β-position of the alcohol used for esterification. *iso*Butyl hexenoate

Table 2. *Yields of esters of α-bromohexanoic acid and of hexenoic acid obtained with α-substituted alcohols*

Ester	Yield of α-bromohexanoate (%)	Yield of hexenoate from hexanoic acid (%)
Methyl	68	0
Ethyl	76	11
*iso*Propyl	63	21
tert.-Butyl	56	6

Table 3. *Yields of esters of α-bromohexanoic acid and of hexenoic acid obtained with β-substituted alcohols*

Ester	Yield of α-bromohexanoate (%)	Yield of hexenoate from hexanoic acid (%)
Ethyl	76	11
n-Propyl	81	19
*iso*Butyl	85	29
Neopentyl	82	49

was formed in comparatively good yield from hexanoic acid by the usual procedure. Still better results were obtained with neopentyl α-bromohexanoate, the overall conversion of hexanoic acid to the hexenoate ester being 49 %. It was interesting to find that benzyl hexenoate could also be obtained in relatively good yield, 24 % overall. All the comparative dehydrobrominations were carried out in quinoline, the results of which are summarized in Tables 1–3. Further experiments with neopentyl α-bromohexanoate showed that diethylaniline was a slightly more effective dehydrobrominating agent

than quinoline or collidine, the latter being the least useful. Diethylaniline is now used as the reagent of choice as the product obtained is also much cleaner than after the use of quinoline. The material produced by dehydrobromination consists largely of the neopentyl ester of the $\alpha\beta$-unsaturated acid (hex-2-enoic acid), but probably also about 8 % of the $\beta\gamma$ isomer (hex-3-enoic acid; cf. Kon, Linstead & Maclennan, 1932). These isomers may be separated by the method of Fittig (1894), but it was found unnecessary in this work, as saponification, followed by fusion of the mixed acids with potassium hydroxide led only to the formation of butyric and acetic acids. No propionic acid was detected, and the $\beta\gamma$ double bond presumably migrated to the $\alpha\beta$-position in a rearrangement similar to that observed with oleic acid (Varrentrap, 1840; Edmed, 1898). Throughout this paper, the name 'hexenoate' refers to the presumed mixture of the $\alpha\beta$ and $\beta\gamma$ isomers.

The mixture of butyric and acetic acids was purified by steam distillation and separated into its component acids by azeotropic distillation, first with benzene and then with toluene (Schicktanz, Steele & Blaisdell, 1940). It was found that the yields were identical with those obtained from pure hex-2-enoic acid. If the ester were fused before purification or saponification, a rather lower yield was obtained. The analysis of the fatty acid mixtures was carried out by paper chromatography (Brown, 1950) and by liquid-vapour partition chromatography (James & Martin, 1951). In order to obtain a pure specimen of acetic acid, it was found necessary to conduct the azeotropic distillation with benzene very slowly, so that maximum equilibrium might be established in the fractionating column. Analysis by liquid-vapour partition chromatography had shown that approximately equimolar quantities of butyric acid and acetic acid were produced during fusion of hexenoic acid with potassium hydroxide. These results were confirmed in the fractionation, each acid being obtained in 40–45 % yield from hexanoic acid.

The butyric and acetic acids obtained could be further degraded by conventional methods (Calvin, Heidelberger, Reid, Tolbert & Yankwich, 1949). [*Carboxy*-^{14}C]-hexanoic acid was now prepared from *n*-amyl magnesium bromide and [^{14}C]-carbon dioxide. Degradation by the above-described method revealed the presence of radioactivity only in the carboxyl carbon of the acetate in theoretical amount, demonstrating the absence of any rearrangement of the carbon atoms in the course of the series of reactions. A description of the application of this new method for the identification of radioactive atoms in hexanoic acid synthesized from [*carboxy*-^{14}C] acetate in the mammary gland of a lactating goat has already been published (French, Hunter, Martin & Popják, 1951; Popják, French, Hunter & Martin, 1951).

The method has been extended to the degradation of octanoic acid with satisfactory results. Neopentyl α-bromo-octanoate was obtained in 96 % yield from octanoic acid, and the corresponding neopentyl octenoate (presumably a mixture of the $\alpha\beta$ and $\beta\gamma$ isomers) in an overall yield of 59 %. Oct-2-enoic acid had previously been prepared by Bourguel (1929) by catalytic reduction of oct-2-inic acid.

Saponification of neopentyl octenoate and fusion of the potassium salt with a further quantity of potassium hydroxide led to the formation of hexanoic acid in high yield, the conversion of octanoic to hexanoic acid proceeding in 51 % overall yield. The yield of acetic acid by azeotropic distillation was, however, considerably smaller (29 % overall), although analysis of the products of fusion by liquid-vapour partition chromatography showed that equimolar quantities of acetic and hexanoic acids were formed. This further revealed the presence of about 5–10 % of octanoic acid which could be separated from the hexanoic acid by azeotropic distillation with xylene.

EXPERIMENTAL

Fusion of hex-2-enoic acid and methyl hex-2-enoate with potassium hydroxide

After hex-2-enoic acid (0·5 g.) had been heated with solid KOH (1·5 g.) at 250° for 1 hr., paper chromatographic analysis of the product revealed unchanged hexenoic acid only. However, when the fusion of hex-2-enoic acid (3·1 g.) was carried out with KOH (10·0 g.) at 300–340° for 5–10 min., chromatographic analysis by the method of James & Martin (1951) demonstrated the formation of equimolar quantities of butyric and acetic acids only. After fusion, the molten mass was allowed to cool, dissolved in a small volume of water and extracted with ether to remove impurities. The aqueous solution was then made acid and steam-distilled. The mixture of acetic and butyric acids thus obtained was neutralized and the solution evaporated to dryness. The two acids were separated by azeotropic distillation (Schicktanz *et al.* 1940) which gave 1·63 g. (81 %) of acetic acid and 1·99 g. (83 %) of butyric acid. Both were pure chromatographically.

Methyl hex-2-enoate was prepared by treating hex-2-enoic acid (10·0 g.) with a solution of diazomethane (5·6 g.) in ether (500 ml.). The mixture was allowed to stand for 24 hr. when the ether and the residual diazomethane were removed by distillation. The residue was taken up in fresh ether (250 ml.) and washed with two portions of water (150 ml. each). The ethereal solution was dried with anhydrous Na_2SO_4, after which the ether was evaporated off. The crude product was distilled at 62–64°/19 mm. to give methyl hex-2-enoate (8·7 g., 78 %). (Found: C, 65·5; H, 9·4. Calc. for $C_7H_{12}O_2$: C, 65·6; H, 9·4 %.) Some unreacted hex-2-enoic acid could be recovered. This ester had previously been prepared by reacting hex-2-enoic acid with methanol in the presence of H_2SO_4 (Baker, Querry, Safir & Bernstein, 1947).

Fusion of methyl hex-2-enoate with KOH at 300–340° gave butyric and acetic acids in a yield similar to that obtained after the fusion of the free acid.

Experiments with α-bromohexanoic acid

α-Bromohexanoic acid was prepared as described in *Organic Syntheses* (1925). This acid (4·0 g.) was treated with diazomethane (2·8 g.) in ether (200 ml.). The reaction took the normal course, and after standing for 12 hr., the ether and residual diazomethane were evaporated off *in vacuo*. The residue distilled at 90–92°/20 mm. yielding methyl α-bromohexanoate (3·5 g., 81 %). (Found: C, 40·2; H, 6·0; Br, 38·4. $C_7H_{13}O_2Br$ requires C, 40·2; H, 6·2; Br, 38·3 %.)

α-Bromohexanoic acid (0·5 g.) was fused with solid KOH (1·0 g.) at 300–340° for 2 hr. Paper chromatographic analysis of the products revealed the presence of pentanoic (valeric) acid, together with a small amount of butyric and acetic acid.

Methyl α-bromohexanoate (10·0 g.) was refluxed with quinoline (30·0 g.) for a few minutes and allowed to cool. The solution was poured into an excess of 4 N-HCl and then extracted with ether. After drying, the ether was evaporated off leaving a crude brown oil (3·5 g.). Distillation at 92°/25 mm. gave the lactide ester of α-hydroxyhexanoic acid (3·2 g., 54 %) as a colourless oil.

[Carboxy-^{14}C]-*hexanoic acid*

[*Carboxy*-^{14}C]-hexanoic acid was prepared from *n*-amyl bromide and $^{14}CO_2$ by the Grignard reaction, the detailed procedure being similar to that used by Anker (1948) for the preparation of (*carboxy*-^{14}C)-acetic acid.

Preparation of alkyl α-bromohexanoates

These esters were all prepared by similar procedures, of which the preparation of *iso*butyl α-bromohexanoate given below may be taken as an example. Hexanoic acid was heated under reflux with a 50 % excess of thionyl chloride for 1 hr.; unreacted thionyl chloride was removed by distillation. Without further purification, the residual hexanoyl chloride was treated with a 5 % excess of dry bromine, and heated under reflux at about 80° until practically all the free bromine had disappeared. A portion of the crude α-bromohexanoyl chloride (15·0 g.) was poured into *iso*butanol (25·0 g.) and the resulting solution allowed to stand for 12 hr. The excess alcohol was removed *in vacuo*, and the residue was distilled at 125–135°/38 mm., yielding *iso*butyl α-bromohexanoate (15·07 g., 85 %) as a colourless oil with a sickly odour. This product was used as such for further stages in the degradation, but if an analytically pure sample was required, the ester was washed with saturated sodium bicarbonate solution and then with distilled water. Redistillation gave a very pure sample. The physical constants and analytical data for the various α-bromohexanoates are listed in Table 4.

Preparation of alkyl hexenoates

The conversion of the esters of α-bromohexanoic acid to the corresponding esters of hexenoic acid is again typified by the example of the *iso*butyl ester given below. *iso*Butyl α-bromohexanoate (14·09 g.) was dissolved in quinoline (72·0 g.) and heated under reflux. When the temperature reached about 180°, the solution deepened in colour to a dark red and the temperature rose almost instantly to the boiling point. The external source of heat was at once removed, and the solution allowed to cool. The excess quinoline was removed by extraction with a twofold excess of 4 N-HCl and separation of the liquid phases. The aqueous solution was extracted with ether, and the extract added to the previously separated crude *iso*butyl hexenoate. After drying, the ether was evaporated off, and the residual brownish oil distilled at 92–98°/19 mm., yielding *iso*butyl hexenoate (3·25 g., 34 %).

Degradation of neopentyl α-bromohexanoate to butyric and acetic acid

Neopentyl α-bromohexanoate (3·89 g.) was heated under reflux with diethylaniline (6·60 g.) for 6 hr. The solution was cooled and poured into a 100 % excess of 20 % (v/v) aqueous H_2SO_4. Extraction with ether, drying and removal of the solvent left a yellow oil. Distillation at 95–96°/18 mm. gave neopentyl hexenoate (1·65 g., 62 %) as a colourless, sweet-smelling liquid. This ester (1·65 g.) was heated under reflux for 1 hr. with a solution of KOH (1·5 g.) in a mixture of ethanol (10 ml.) and water (5 ml.). The solution was evaporated to dryness on the steam bath and a further quantity of KOH (4·5 g.) added. Fusion at 300–340° was carried out for 10 min., and the molten mass was allowed to cool before extraction with water (30 ml.). The aqueous solution was extracted with ether to remove impurities, and then acidified with H_2SO_4. Steam distillation was then carried out, and the distillate was shown by liquid-vapour partition chromatography to contain equimolar quantities of acetic and butyric acid. The distillate required 15·90 ml. of N-NaOH for neutralization, equivalent to a yield of 89 %, similar to that obtained by fusion of pure hex-2-enoic acid with KOH. Direct alkaline fusion of unsaponified neopentyl hexenoate or of the crude undistilled ester gave lower yields (approx. 75 %).

Degradation of octanoic acid to hexanoic acid

Octanoic acid (10·0 g.) was heated under reflux with thionyl chloride (14 ml.) for 1 hr. The excess of reagent was removed by distillation through a small column at atmo-

Table 4. *Physical constants and analytical data of esters of α-bromohexanoic acid*

Ester	b.p.	Refractive index	Analysis: Found (%) C	H	Br	Requires (%) C	H	Br
Ethyl	120–122°/41 mm.	n_D^{20} 1·4509	—	—	—	—	—	—
*iso*Propyl	100–101°/25 mm.	n_D^{20} 1·4432	45·4	6·9	34·0	45·6	7·20	33·80
tert.-Butyl	103–104°/18 mm.	n_D^{20} 1·4682	48·7	7·7	32·0	47·8	7·60	31·90
n-Propyl	110–111°/20 mm.	n_D^{22} 1·4569	45·2	6·7	33·2	45·6	7·15	33·75
n-Capryl	160–161°/18 mm.	n_D^{18} 1·4534	54·5	8·7	25·8	54·7	8·80	26·10
*iso*Butyl	115–116°/22 mm.	n_D^{20} 1·4356	47·6	7·9	32·1	47·8	7·60	31·90
Neopentyl	120–122°/20 mm.	n_D^{23} 1·4530	49·5	7·9	30·5	49·8	7·90	30·20
Benzyl	124–125°/0·4 mm.	n_D^{23} 1·5253	—	—	27·5	—	—	28·05

spheric pressure, and bromine (3·8 ml., dried with conc. H_2SO_4) was added to the residue. After heating under reflux at 80–100° for a further 2–3 hr., by which time practically all the free bromine had disappeared, the product was added to a threefold excess of neopentyl alcohol. The dark brown solution obtained was allowed to stand for several hours before removing the excess of neopentanol under reduced pressure. The residue distilled at 148–155°/22 mm., yielding *neopentyl α-bromo-octanoate* (19·53 g., 95%). Analytically pure material was obtained by washing with saturated aqueous $NaHCO_3$, extraction with ether and drying over anhydrous Na_2SO_4. After removal of the ether, distillation at 89°/0·5 mm. gave the pure ester. (Found: C, 53·1, H, 8·8; Br, 27·0. $C_{13}H_{25}O_2Br$ requires C, 53·2; H, 8·5; Br, 27·3%.) For degradative purposes the once-distilled material proved satisfactory, as was the case with neopentyl α-bromohexanoate. Neopentyl α-bromo-octanoate (13·58 g.) was treated with diethylaniline (21·0 g.) and heated under reflux for 6 hr. The solution was then only slightly discoloured. It was poured into a large excess of 20% H_2SO_4 to remove the residual diethylaniline and the resulting aqueous solution was extracted three times with an equal volume of ether. The ethereal extract was dried over anhydrous Na_2SO_4, and the ether then removed. The residue was distilled at 124–127°/19 mm., giving *neopentyl octenoate* (6·05 g., 62%) as a colourless, sweet-smelling oil. The ester was further purified with only trivial loss by redistillation at 118–120°/18 mm. (Found: C, 74·0; H, 11·4. $C_{13}H_{24}O_2$ requires C, 73·6; H, 11·3%.)

Neopentyl octenoate (0·48 g.) was dissolved in ethanol (3 ml.) and a solution of KOH (0·5 g.) in water (1·5 ml.) added. The mixture was heated under reflux for 2 hr. and evaporated to dryness on the steam bath. A further quantity of KOH (2·0 g.) was added, and fusion was then carried out at 300–350° until gas evolution practically ceased. The product was dissolved in water (30 ml.) and non-acidic impurities removed by ether extraction. The aqueous solution was now neutralized with H_2SO_4. Vapour phase chromatography revealed the presence of equivalent amounts of acetic and hexanoic acid, but also a smaller quantity (5–10% of the hexanoic acid) of octanoic acid. The fusion was repeated under the same conditions with a further portion of neopentyl octenoate (1·00 g.), but this time the acidified aqueous extract was steam distilled. The mixed acids in the steam distillate were neutralized, and the water then evaporated off. The free acids were liberated from the salts by treatment with a large excess of *p*-toluenesulphonic acid in dry benzene, and fractionated by azeotropic distillation, first with benzene, and then with xylene, any octanoic acid remaining behind. Chromatographically pure acetic acid (0·131 g., 46%) and hexanoic acid (0·465 g., 85%) were obtained. Thus the overall conversion of octanoic to hexanoic acid was 51%.

SUMMARY

1. A new method for the degradation of *n*-carboxylic acids is described in which two carbon atoms are removed from the carboxyl end as acetic acid, the other product being a *n*-carboxylic acid with two carbon atoms less than the original acid.

2. The chemical reactions involved in the degradation are: (i) conversion of the carboxylic acid to the corresponding acid chloride; (ii) bromination of the acid chloride; (iii) conversion of the α-bromo acid chloride to an ester by reaction with an alcohol; (iv) dehydrobromination of the resulting α-bromo ester; and (v) saponification of the unsaturated ester produced in reaction (iv), and fusion of the salt with solid potassium hydroxide.

3. Hexanoic acid was used in model experiments, and wide variation was found in the yields obtained in the dehydrobromination stage when different esters were used. The neopentyl ester gave the best yield.

4. Diethylaniline was found to be a more satisfactory reagent for the dehydrobromination of the α-bromo esters than either quinoline or collidine.

5. [*Carboxy*-^{14}C]-hexanoic acid has been synthesized, and the degradation carried out on this labelled material. Radioactive carbon was found only in the carboxyl carbon of the acetic acid obtained by the degradation indicating that rearrangement of the carbon atoms did not occur during the reactions.

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Isolation of Antibiotics from a Species of *Cephalosporium*. Cephalosporins P_1, P_2, P_3, P_4 and P_5

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Brotzu (1948) reported that a species of *Cephalosporium* isolated from the sea near Sardinia produced a substance that inhibited the growth of a number of Gram-positive and Gram-negative bacteria. The production of antibacterial substances by the same *Cephalosporium* has recently been studied in this laboratory and in the Antibiotics Research Station of the Medical Research Council at Clevedon (Crawford, Heatley, Boyd, Hale, Kelly, Miller & Smith, 1951). This paper describes the isolation and some of the properties of members of one group of antibiotics obtained from active culture filtrates of the organism.

At an early stage of the present work it was found that some culture fluids contained at least two kinds of antibacterial substance, one of which could readily be extracted into butyl acetate from a weakly acid solution while the other was insoluble in common organic solvents. The active material in the ester-soluble fraction, with which this paper is concerned, was called cephalosporin P. The remaining water-soluble substance, which was discovered independently at Clevedon, had quite different chemical and antibacterial properties. It was named cephalosporin N and will be the subject of a separate publication. Solutions of cephalosporin P inhibit the growth of *Staphylococcus aureus* but not of *Salmonella typhi*, whereas cephalosporin N is active against both organisms. It is probable that the crude extracts prepared by Brotzu (1948) contained mainly cephalosporin N, while the material studied in earlier work at Oxford (Crawford *et al.* 1951), which could be extracted by organic solvents, was cephalosporin P.

It has now been shown that crude cephalosporin P contains five acidic antibiotics. These substances have been named cephalosporins P_1, P_2, P_3, P_4 and P_5. Cephalosporins P_1, P_2 and P_4 have been isolated in crystalline form. All five substances have been found to have some properties in common with helvolic acid, an antibiotic produced by various strains of *Aspergillus fumigatus* (Chain, Florey, Jennings & Williams, 1943).

Ritchie, Smith & Florey (1951) have shown that cephalosporin P_1 is almost as active as aureomycin and terramycin against the staphylococcus *in vitro*, and that it is less toxic than these substances when given intravenously and can readily be obtained in the blood in bacteriostatic concentration. For reasons that are not yet understood, however, cephalosporin P_1 has proved much less effective than aureomycin or terramycin in curing staphylococcal infections in mice.

Isolation of cephalosporins P_1, P_2 and P_4

Culture fluids obtained from the Antibiotics Research Station at Clevedon contained much larger amounts of cephalosporin P_1 than of other components of the P complex, and the process of purification was directed towards the isolation of this substance. The minor active constituents occurred in variable amounts in different batches of material. Information concerning cephalosporins P_2, P_3, P_4 and P_5 was accumulated in the course of the work, during which the various fractions obtained were examined by paper chromatography. The cylinder-plate method of assay (Heatley, 1944), with *Staph. aureus* as a test organism, was used to follow the active substances.

The acidic cephalosporin P could be extracted almost completely from an aqueous solution at pH 6·5 into one-fifth of a volume of butyl acetate, and since the extract could be concentrated by distillation without loss of activity this procedure was used for removing the antibiotics from large

batches of culture fluid. Re-extraction of cephalosporin P_1 into aqueous solution in the form of a salt proved unsatisfactory, because the partition coefficient did not greatly favour the aqueous phase until the pH was raised to a value at which inactivation began to occur. A preliminary purification was therefore carried out by precipitations from mixed organic solvents, making use of the fact that the active material was very soluble in acetone, moderately soluble in di*iso*propyl ether, and insoluble in hexane or light petroleum. The resulting material was then purified further by counter-current distribution between solvents and by chromatography.

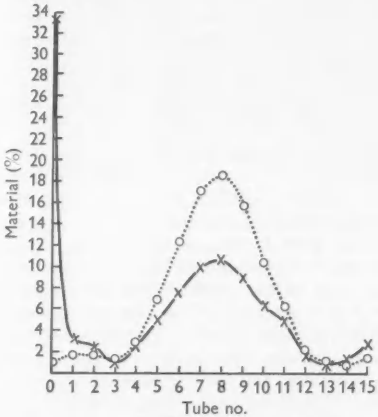

Fig. 1. 15-Transfer distribution of partly purified cephalosporin P in a system composed of hexane-di*iso*propyl ether-acetone-0·5 M-potassium phosphate pH 6·0 (25:8:25:25, by vol.). Lower phase mobile. O··O··O, Percentage total activity; ×—×—×, percentage total weight.

Counter-current distributions were carried out in a solvent system composed of hexane-di*iso*propyl ether-acetone-M-potassium phosphate, pH 6·0 (1:0·3:1:1, by vol.), the lower phase being mobile. In this system the overall partition coefficient of the active material was close to unity. A distribution curve obtained with one batch of material, which had been subjected to fifteen transfers, is shown in Fig. 1. Most of the activity was present in the central fractions, while most of the impurities were contained in fractions at either end of the series together with minor active components of the mixture.

With a few batches of culture fluid the material recovered from fractions 5–10 of the distribution was almost pure cephalosporin P_1, which could be crystallized directly from aqueous methanol. Of the relatively small amount of remaining active material a high proportion of the total quantity of cephalosporin P_3 was contained in fractions 14 and 15, while cephalosporin P_5 was concentrated in fractions 0–5.

With most batches of culture fluid the most active material obtained by counter-current distribution needed further purification before it would yield crystalline cephalosporin P_1. This material was chromatographed from di*iso*propyl ether on a column of Florisil (magnesium silicate). By

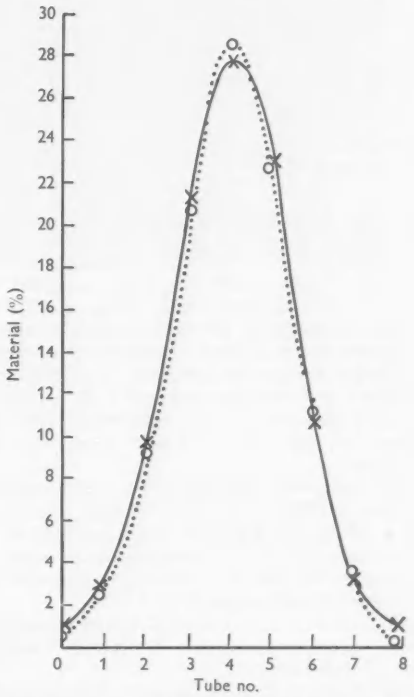

Fig. 2. 8-Transfer distribution of cephalosporin P_1 in a system composed of hexane-di*iso*propyl ether-acetone-0·5 M-potassium phosphate pH 6·0 (25:8:25:25, by vol.). Lower phase mobile. O··O··O, Percentage total activity; ×—×—×, percentage total weight.

developing successively with solvents of increasing polarity, such as di*iso*propyl ether, ethereal acetone, acetone, and acetone containing 1% of water, different constituents of the material passed as bands down the column and were collected in the percolate. The band containing most of the activity normally emerged from the column during elution with di*iso*propyl ether-acetone. This fraction consisted of cephalosporin P_1, together with a small amount of cephalosporin P_2. Elution with acetone

then removed any cephalosporin P_4. Cephalosporin P_3 was absorbed more strongly than the other antibiotics, and was only removed during the final elution with aqueous acetone.

The purest material in the main active band from the column behaved as though it were a single substance in an eight-transfer counter-current distribution. Fig. 2 shows the result of such a distribution, in which the experimental curve agrees closely with that expected for one substance with a partition coefficient of 1·05. Nevertheless, the material was not always homogeneous. On dissolving its main constituent, cephalosporin P_1, in di*iso*propyl ether, a small residue of less soluble material sometimes remained which consisted of cephalosporin P_2.

The purest preparation of cephalosporin P_1 crystallized readily in needles from aqueous methanol. Cephalosporins P_2 and P_4 crystallized from the same solvent.

Properties of cephalosporins P_1, P_2, P_3 and P_4

Cephalosporin P_1. Crystalline cephalosporin P_1, m.p. 147° (micro block), $[\alpha]_D + 28°$, analysed as follows: C, 65·8; H, 9·0; *C*-CH_3, 12·1; acetyl 16·8; active hydrogen 0·6 %. Nitrogen, halogens, sulphur, and phosphorus were absent. The equivalent weight by titration, using phenolphthalein as an indicator, was 580. On electrometric titration in 60 % ethanol the substance behaved as a monobasic acid. It appears to have a polycyclic hydroaromatic structure.

Cephalosporin P_1 is readily soluble in most organic solvents, but sparingly soluble in light petroleum and in water. It is stable in organic solvents, or as the sodium salt at room temperature in aqueous solution at pH 7·0, but is slowly inactivated at pH 8·0 and rapidly at pH 9·6.

When tested by the cylinder-plate method against *Staph. aureus*, cephalosporin P_1 is nearly twice as active as helvolic acid.

Cephalosporin P_2. Crystalline cephalosporin P_2, m.p. 151° (micro block), gave the following results on analysis: C, 65·25; H, 8·5; *C*-CH_3, 9·4; OCH_3, 5·8; acetyl, 18·8; active hydrogen 0·8 %.

The substance is an acid readily soluble in ethanol, acetone, and butyl acetate, but much less soluble than cephalosporin P_1 in di*iso*propyl ether or benzene. When tested by the cylinder-plate method against *Staph. aureus* its activity was similar to that of cephalosporin P_1.

Cephalosporin P_3. This antibiotic was less stable than the other cephalosporins and was not isolated in the crystalline state. It is an acidic substance, readily soluble in most organic solvents, but sparingly soluble in water. It formed a white greasy solid with an activity against *Staph. aureus* similar to that of cephalosporin P_1, but rapidly became inactive, brown-coloured, and insoluble in acetone, on standing in air.

Cephalosporin P_4. This acidic antibiotic formed light-fawn coloured crystals, m.p. 220–230° (micro block) after one recrystallization from aqueous methanol. It is readily soluble in acetone, but sparingly soluble in water or ether. In ethereal solution it lost no activity in 3 months at room temperature.

Inactivation of cephalosporin P by a preparation of penicillinase

Early in the purification of cephalosporin P crude material extracted from the culture fluid was found to be inactivated by a sample of penicillinase prepared from a strain of *Bacillus subtilis* by the method of Duthie (1944). When purified cephalosporins became available the compounds P_1, P_2, P_3, P_4 and P_5 were tested individually and found to lose activity in the presence of the same enzyme preparation.

Since the cephalosporins have no chemical relationship to penicillin it seemed unlikely that they would be inactivated by penicillinase itself. Crawford (1951) has now found that several strains of aerobic spore-forming organisms produce a substance able to inactivate cephalosporin P_1, and that the amount of the substance in different culture fluids is not proportional to the amount of penicillinase. It may therefore be concluded that an impurity in the preparation of penicillinase is responsible for the inactivation of the cephalosporins. The inactivating agent, which is heat-labile and non-dialysable, appears to be an enzyme, or group of enzymes, and has provisionally been called P-cephalosporinase.

Behaviour of the cephalosporins on paper chromatograms

Cephalosporin P_5. The behaviour of the various cephalosporins on paper chromatograms was examined during the course of the purification, and compared with that of helvolic acid. Three solvent systems were used: (1) 0·02 N-acetic acid in 25 % (v/v) ethanol. (2) Di*iso*propyl ether saturated with 0·1 M-potassium phosphate, pH 7·0. (3) Amyl acetate saturated with 0·1 M-potassium phosphate pH 7·0. With systems (2) and (3) the paper was treated with M-potassium phosphate pH 7·0 and dried before use. After development the position of the active spots was determined by placing the paper on plates seeded with *Staph. aureus*. The results given in Table 1 show that the chromatograms sharply differentiate cephalosporin P_5 from P_1, P_2 and P_4, and that they provide evidence for the existence of a fifth antibiotic, cephalosporin P_5. In the countercurrent distribution (Fig. 1) cephalosporin P_5 was found mainly in fractions 0–5.

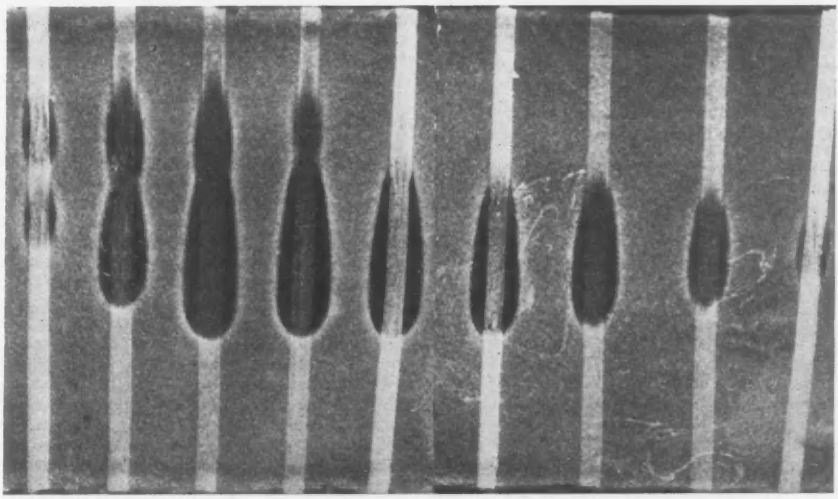

Fig. 3. Paper chromatograms of fractions from an 8-transfer distribution of crude cephalosporin P enriched in cephalosporin P_5. The solvent was 25% ethanol in 0·02 N-acetic acid. The position of the antibiotics is marked by clear zones on an agar plate seeded with *Staph. aureus*. All the fractions contain cephalosporin P_1 and fractions 0–3 also contain cephalosporin P_5.

Table 1. *Chromatography of cephalosporins*

Antibiotic	R_F values in System 1*	System 2*	System 3*	Other properties
P_1	0·45	0·50	1·0	M.p. 147°. Soluble in di*iso*propyl ether
P_2	0·45	—	—	M.p. 151°. Almost insoluble in di*iso*propyl ether
P_3	0	0	<0·05	Unstable in the solid state
P_4	0·40	0·50	1·0	M.p. 220–230°
P_5	0·20	—	—	—
Helvolic acid	0	0·6	1·0	M.p. 212–220°

* See text.

Fig. 3 shows chromatograms of the fractions from an eight-transfer counter-current distribution of material already concentrated with respect to cephalosporin P_5. All the fractions contain cephalosporin P_1. Cephalosporin P_5 is absent from fractions 5–9 and its concentration relative to cephalosporin P_1 diminishes from fraction 0 to fraction 4.

By comparing the R_F values and other properties of the antibiotics (Table 1), it becomes clear that cephalosporins P_1, P_2, P_3, P_4 and P_5 are all separate compounds, and that they are all different from helvolic acid.

Relationship of cephalosporin P_1 to helvolic acid

During a preliminary examination of crude cephalosporin P it was noticed that some of the physical properties of this material resembled those of helvolic acid. Later, a resemblance was found between the antibacterial range and also the pharmacological properties of helvolic acid and cephalosporin P_1 (Ritchie *et al.* 1951). This resemblance was heightened by two further observations: (1) helvolic acid, like cephalosporins P_1, P_2, P_3, P_4 and P_5, was inactivated by incubation with a preparation of penicillinase from a strain of *B. subtilis*; (2) *Staph. aureus* became resistant to cephalosporin P_1 as readily as it did to helvolic acid (Abraham, Callow & Gilliver, 1946). Staphylococci that had become resistant to helvolic acid by growth in the presence of this substance were found to have also become resistant to cephalosporin P_1. Conversely, organisms that were grown in the presence of cephalosporin P_1 developed resistance to helvolic acid as well as to the cephalosporin. These pro-

perties suggest that the antibacterial activity of helvolic acid and cephalosporin P depends on a similar structural feature.

Cephalosporins P_1 and P_2, and helvolic acid ($C_{32}H_{44}O_8$), have rather similar molecular formulae. Further information about the chemical nature of these substances will be given in a future publication.

EXPERIMENTAL

Method of isolation

Extraction into butyl acetate. The filtered culture fluid, prepared at the Antibiotics Research Station, Clevedon, (Crawford *et al.* 1951), was adjusted to pH 6·5 by the addition of 10 N-HCl and stirred for 5 min. with one-fifth of its volume of butyl acetate. The two phases were separated in a Sharples centrifuge. Over 95 % of the cephalosporin P was extracted into the organic solvent.

Concentration. The butyl acetate extract (40 l.) was concentrated *in vacuo* to 2 l. using a laboratory climbing-film still (Van Heyningen, 1949). Less than 5 % of the activity was lost in this operation. The concentrate was then reduced to 200 ml. by distillation *in vacuo* (bath temperature 55°) from a 5 l. flask.

With some batches of material a white solid separated from the concentrated butyl acetate on standing and was removed. This substance separated from aqueous acetone or ethanol in the form of white crystals, m.p. 236°, and analysed as follows: C, 70·2; H, 9·0; *C*-CH_3, 9·4; acetyl, 9·0 %. It was an acidic compound which was sparingly soluble in water and less soluble in acetone than the cephalosporins. When tested by the cylinder-plate method against *Staph. aureus* at pH 7·0 it showed no activity at a concentration of 1 mg./ml.

Precipitation with light petroleum. To the concentrate (200 ml.) was added 2 l. of light petroleum (b.p. 100–120°) with stirring. A precipitate formed which contained 80 % of the active material but only 60 % of the total solids.

Precipitation with diisopropyl ether. The active precipitate was separated by centrifuging and dissolved in acetone (100 ml.). Di*iso*propyl ether (1 l.) was added with stirring to the acetone. An almost inactive precipitate formed which contained nearly 20 % of the total material. This was removed at the centrifuge. About 97 % of the active material remained in solution.

Precipitation by concentration in diisopropyl ether-light petroleum. The crude cephalosporin in di*iso*propyl ether (1 l.) was mixed with an equal volume of light petroleum (b.p. 100–120°). An almost inactive precipitate was removed. The ratio of light petroleum to di*iso*propyl ether was then increased by concentrating the solution *in vacuo* to 750 ml., when most of the cephalosporin P was precipitated. The precipitate at this stage contained about 50 % of the total activity in the culture fluid and was about half as active against *Staph. aureus* as pure cephalosporin P_1.

Transfer between solvents. With some batches of material, containing large amounts of inactive material, further purification was carried out in the following manner before undertaking a counter-current distribution between solvents. If this were not done large volumes of solvent were necessary in the distribution to avoid the separation of the system into three phases.

The crude precipitate was dissolved in acetone (50 ml.), di*iso*propyl ether (1·3 l.) and hexane (900 ml.) were added, and the mixture was shaken twice with one-tenth of its volume of M-potassium phosphate, pH 7·0, the pH being kept constant by the addition of a few drops of conc. NaOH. About 85 % of the activity remained in the less polar phase. The aqueous phase, containing much inactive material, was rejected.

The solvent was removed from the less polar phase *in vacuo*, the residue dissolved in acetone (1100 ml.) and the solution mixed with an equal volume of M-potassium phosphate, pH 7·0. The acetone-phosphate buffer was shaken with 1/20 vol. of a mixture of hexane-di*iso*propyl ether (3:2). About 90 % of the activity remained in the aqueous acetone. The latter was concentrated *in vacuo* to 1500 ml., centrifuged clear, and acidified to pH 2·0, when the cephalosporin P was precipitated.

Counter-current distribution between solvents. The crude cephalosporin (11 g.) was subjected to fifteen transfers in a system composed of hexane-di*iso*propyl ether-acetone-0·5 M-potassium phosphate, pH 6·0 (25:8:25:25, by vol.). The transfers were carried out in separating funnels containing 240 ml. of each phase, and the lower phase was mobile. At the end of the experiment the active material in each lower phase was removed by three extractions with a mixture of ether (46 ml.) and light petroleum (b.p. 60–80°; 23 ml.) and the extracts were added to the corresponding upper phases. Samples of the resulting solutions were used to determine the amount of material in each tube. The samples (1–5 ml.) were placed in small weighed test tubes held in a metal block. The solvent was evaporated in a stream of dry air, the block being finally heated in a water bath to 80°, and the tubes were then reweighed. The results of one experiment are shown in Fig. 1.

Crystallization of cephalosporin P_1. The main quantity of material in fractions 5–10 was recovered by evaporating the appropriate solutions *in vacuo* to dryness. The resulting white solid could be crystallized from aqueous methanol and after three crystallizations yielded pure cephalosporin P_1, m.p. 145° (4 g.).

Chromatography on Florisil. With some batches of culture fluid the most active material obtained from the counter-current distribution did not yield crystalline cephalosporin P_1. In these cases the material was purified further by chromatography on Florisil (magnesium silicate, provided by the Floridin Company, Warren, Pa., U.S.A.) or on alumina (pH 5). The active constituents of the various fractions were analysed by paper chromatography. The precise behaviour of the active material on the Florisil chromatograms varied with the loading of the column and the impurities present. The following is a typical experiment.

A solution of impure cephalosporin (1·44 g.) in di*iso*propyl ether was run on to a column of Florisil (100–200 mesh) 29 cm. long and 2 cm. in diameter. The chromatogram was developed with a series of solvents of increasing polarity and the eluate was collected in 100 ml. fractions, samples of which were used to determine activity and dry weight. Development with di*iso*propyl ether (300 ml.) resulted in the elution of inactive material (300 mg.). Further development with diethyl ether (750 ml.) removed most of the cephalosporin P_1 and P_2 (380 mg.). Slightly less active material, containing cephalosporin P_4, was then eluted with acetone (50 ml.), having moved down the column as a sharp yellow band. Finally, material containing cephalosporin P_3 was eluted with acetone containing 1 % (by vol.) of water.

The major active fraction (380 mg.) was rechromatographed on a column of Florisil 20 cm. long and 1·7 cm. in diameter. Elution with diethyl ether (735 ml.) removed 125 mg. of inactive material. Subsequent elution with acetone (600 ml.) removed 132 mg. of almost homogeneous cephalosporin P_1 containing a little cephalosporin P_2. A final elution with acetone containing 1 % of water by vol. removed 120 mg. of almost inactive material. Cephalosporin P_1 was dissolved in di*iso*propyl ether (50 ml.) and the solution centrifuged from a small amount of cephalosporin P_2. After evaporation of the ethereal solution the remaining cephalosporin P_1 was crystallized from aqueous methanol.

The fraction from the first column which contained cephalopsorin P_2 was rechromatographed, the same series of solvents as before being used for development. The cephalosporin P_2 was again eluted by aqueous acetone. On the other hand, when active material containing no cephalosporin P_2 was rechromatographed no activity remained on the column at this stage.

The fraction containing cephalosporin P_4 was concentrated to about 5 ml. Ether (20 ml.) was then added and the mixture allowed to stand at 0°. Cephalosporin P_4 separated in crystalline form and was recrystallized from aqueous methanol.

Paper chromatography. Chromatograms on Whatman no. 1 paper (42 × 23 cm.) were carried out in large accumulator jars (42 cm. high), the tops of which (26 × 23 cm.) had been ground flat and sealed with glass lids. The jars carried a stainless steel trough supported on a stainless steel frame. Three systems were used for development: (1) ethanol-0·1 N-acetic acid-water (2·5:2·0:5·5, by vol.), (2) di*iso*propyl ether saturated with 0·1 M-potassium phosphate, pH 7·0, (3) amyl acetate saturated with 0·1 M-potassium phosphate, pH 7·0.

The paper was treated with M-potassium phosphate buffer, pH 7·0 before use with systems (2) and (3). After being dipped in the buffer solution it was pressed between sheets of filter paper and then dried in air.

Samples for analysis, containing 10–20 μg. of antibiotic in 5 μl. of acetone or di*iso*propyl ether, were placed near the top of the paper sheet in a series of spots 2·5 cm. apart. The chromatograms were run for about 5 hr. at 24°, after which the paper was dried in air. The paper was then cut into strips, 1 cm. wide, containing the material from the different samples, and the strips were placed for 30 min. on large plates of agar bulk-seeded with *Staph. aureus* (National Collection of Type Cultures (N.C.T.C.) No. 6571). The strips were then removed and the plates incubated for 18 hr. at 37°, when clear zones in which no growth had occurred marked the positions of the active spots.

Acquired resistance of Staph. aureus *to helvolic acid and cephalosporin P_1.* (*a*) An aqueous solution of the Na salt of helvolic acid (0·04 mg. in 0·04 ml.) was added to 10 ml. of sterile broth in an Erlenmeyer flask, giving a concentration of 4 μg./ml. The broth was inoculated with three drops of an 18 hr. culture of *Staph. aureus*, N.C.T.C. No. 6571 (about 30×10^6 cells), and the flask placed on a shaker at 37°. There was no visible growth in 6 hr., but heavy growth in 24 hr. Two drops of this culture were used to inoculate 10 ml. of broth in a second flask containing helvolic acid at a concentration of 50 μg./ml. There was heavy growth after overnight incubation. Assay plates were seeded with an appropriate dilution of this culture, and with a subculture obtained from it that had grown in normal broth. When tested on the plates by the cylinder-plate method, neither helvolic acid nor cephalosporin P_1 gave a measurable zone of inhibition at a concentration of 40 μg./ml. When tested on a comparable plate seeded with a culture of the original staphylococcus, cephalosporin P_1 gave zones of 32 and 21 mm. diameter at concentrations of 40 and 10 μg./ml. respectively, while helvolic acid gave zones of 29 and 18 mm. at the same concentrations.

(*b*) A culture of *Staph. aureus* resistant to cephalosporin P_1 was prepared in a manner similar to that described for helvolic acid. When tested on plates seeded with this culture, neither cephalosporin P_1 nor helvolic acid gave measurable zones of inhibition at a concentration of 40 μg./ml.

Inactivation by a preparation of penicillinase. (*a*) Four 0·5 ml. samples of a solution of cephalosporin P_1 (10 μg.) in 0·1 M-potassium phosphate, pH 7·0, were mixed with 0·5 ml. of solutions of penicillinase in water containing 125, 31, 7·8 and 1·9 μg., respectively, of the enzyme preparation. The penicillinase was a preparation made by Duthie (1944). The mixtures were incubated for 2 hr. at 37° and then assayed by the cylinder-plate method using *Staph. aureus* as a test organism. The results were as follows:

Penicillinase preparation (μg./ml.)	125	31	7·8	1·9
Loss of activity (%)	100	65	20	10

Similar results were obtained with cephalosporins P_2, P_3, P_4, and P_5.

(*b*) One 0·5 ml. sample of a solution of crude cephalosporin P_1 (20 μg.) in 0·1 M-potassium phosphate, pH 7·0, was mixed with 0·5 ml. of a solution of the penicillinase preparation (500 μg.) in water, and a second sample with a similar solution of 'penicillinase' that had been heated for 10 min. at 100°. Both mixtures were incubated at 37° for 2 hr. The cephalosporin in the first solution was completely inactivated, while that in the second solution showed no loss of activity.

(*c*) A solution of the penicillinase preparation (10 mg.) in distilled water (10 ml.) was placed in a cellophan tube and dialysed for 18 hr. at 2° against distilled water (10 ml.). The tube was slowly rotated throughout the dialysis to stir the liquid inside and outside the membrane. The dialysate and the solution inside the tube were then tested separately against cephalosporin P_1. When 0·5 ml. of the dialysate was incubated at 37° with 0·5 ml. of a solution of cephalosporin P_1 (5 μg.) the antibiotic lost no activity. When 0·25 ml. of the solution remaining inside the cellophan tube was incubated at 37° with 0·75 ml. of a solution of cephalosporin P_1 (5 μg.) there was complete loss of activity.

(*d*) A solution of helvolic acid (40 μg./ml.) in 0·1 M-potassium phosphate buffer, pH 7·0, containing the penicillinase preparation (500 μg./ml.) was incubated at 37° for 2 hr. No activity against *Staph. aureus* could be detected by the cylinder-plate method. A control solution incubated in the absence of penicillinase lost no activity.

Effect of metal ions and cysteine. Solutions (1 ml.) of the Na salt of cephalosporin P_1 (10 μg./ml.) were incubated for 2 hr. at 37° in the presence of 1 mg. each of $CuSO_4.5H_2O$, $ZnSO_4$, $AgNO_3$, $Pb(NO_3)_2$, $HgCl_2$, and cysteine. There was no loss of activity.

SUMMARY

1. A species of *Cephalosporium* produces a group of antibiotics that have been called collectively cephalosporin P, and an antibiotic with entirely

different properties which has been named cephalosporin N. Cephalosporin P consists of acidic substances that are soluble in organic solvents, and are active mainly against Gram-positive organisms. Cephalosporin N cannot be extracted from aqueous solution by common organic solvents and is active against both Gram-positive and Gram-negative organisms.

2. Crude cephalosporin P can contain at least five antibiotics, which have been named cephalosporin P_1, P_2, P_3, P_4 and P_5. Cephalosporin P_1 was the major active substance in extracts of the culture fluids that were used in the present work. By counter-current distribution between solvents and chromatography on Florisil, cephalosporins P_1, P_2 and P_4 have been isolated in crystalline form.

3. Cephalosporin P_1 is almost twice as active as helvolic acid against *Staph. aureus*, but the two antibiotics resemble each other in certain chemical and biological properties. Staphylococci that acquire resistance to cephalosporin P_1 by growing in its presence become, at the same time, resistant to helvolic acid. Similarly, resistance acquired to helvolic acid is accompanied by an increase in resistance to the cephalosporin. Cephalosporin P_1 is inactivated by a substance that is present in a preparation of penicillinase. This substance has been provisionally named P-cephalosporinase. The same preparation of penicillinase also causes the inactivation of cephalosporins P_2, P_3, P_4, P_5, and helvolic acid.

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Tissue Fractionation Studies

1. THE EXISTENCE OF A MITOCHONDRIA-LINKED, ENZYMICALLY INACTIVE FORM OF ACID PHOSPHATASE IN RAT-LIVER TISSUE

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In the course of an investigation on the specific glucose-6-phosphatase of liver, the results of which have been published elsewhere (Duve, Berthet, Hers & Dupret, 1949; Hers, Berthet, Berthet & Duve, 1951), some peculiar observations were made on the unspecific acid phosphatase which is also present in hepatic tissue. Evidence was obtained, indicating that this enzyme is associated with mitochondrial granules by some kind of labile linkage, which renders it inactive towards added glycerophosphate at pH 5.

Experiments which substantiate this conclusion are reported in the present paper. A short account of these findings has already been published in preliminary form (Duve, Berthet, Berthet & Appelmans, 1951).

METHODS

Fractionation of tissue constituents. Rats, fasted for 12 hr. were killed by a blow on the head and bled, and the liver taken out immediately and immersed in an ice-cold isotonic solution. In most cases, the chilled organ was pressed through a wire mesh (Claude, 1946), in order to remove the main vascular and connective network, and the resulting pulp was weighed and homogenized in cold 0·25 M-sucrose, using a Pyrex glass homogenizer of the Potter-Elvehjem type (Potter & Elvehjem, 1936).

In the subsequent fractionation, the technique of Claude (1946), as modified by Schneider (1948), was followed in all

essential details. The homogenate was first centrifuged 10 min. at 600 *g* in the Potter-Elvehjem tube. After decanting the supernatant, the precipitate, containing, in addition to red blood-cells, unbroken liver cells and gross cytoplasmic debris, all the nuclei and part of the mitochondria, was rehomogenized with a new quantity of fluid and recentrifuged. This procedure was repeated a second time. The final precipitate, which contained no intact cells or coarse cellular debris and was free of most of the mitochondria, represented the nuclei fraction. The combined supernatants were used for the isolation of the other fractions.

To reconstitute the initial homogenate, samples of the combined supernatants and of the nuclear fraction, representing the same amount of original tissue, were mixed together, furnishing a more reliable preparation than the actual first homogenate, in which all the cells were not entirely broken up.

The mitochondria were separated from the supernatants of the first centrifugations by centrifuging 10 min. at 8500 *g*, using lucite tubes and an MSE 'Major' (Measuring and Scientific Equipment Ltd., London, S.W. 1) centrifuge with multispeed attachment. The supernatant was removed carefully with a medicine dropper. The precipitate was resuspended in a new quantity of fluid by means of a motor-driven glass pestle, whose extremity had been blown into a spherical bulb fitting closely into the bottom of the centrifuge tube. The mitochondria were washed either once or twice. When washed mitochondria were prepared, the procedure was stopped here.

In complete fractionations, the supernatant and washings of the preceding centrifugations were combined and centrifuged for 90 min. at 20,000 *g* to separate the microsomes. The precipitate was washed once or twice, using the same technique as that described for washing the mitochondria. By combining the supernatant and washings of these centrifugations, the final supernatant was obtained.

Samples of each fraction were kept for enzyme assays. In making up the volumes of the various fractions, care was taken to render them all comparable with each other and with the initial homogenate, in order to allow a quantitative study of the enzyme distribution to be made.

The entire fractionation was carried out in the cold room. In order to keep the temperature as low as possible during the high-speed centrifugations, the centrifuge was allowed to run with the lid open in a room at $-5°$. Despite this precaution, the temperature of the rotor reached 10° at the end of the long runs at 20,000 *g*. The gravitational field strengths given are for the middle of the centrifuge tubes.

Assay of enzyme activity. The enzymic tests were performed at pH 5 and 38°, using commercial β-glycerophosphate (Judex) as substrate. In most experiments, a universal phosphate free buffer was used, containing equivalent amounts of sodium acetate, cacodylate and borate, and adjusted with HCl (*ABC* buffer; Duve *et al.* 1949). These buffer mixtures, which cover the pH range from 4 to 10, have been used in this laboratory to study a number of enzymes, including several phosphatases, phosphorylase, phosphoglucomutase, hexokinase and fructokinase. They exert few inhibitions and are easily prepared from stock solutions which keep indefinitely. Their use in this work, which started more or less fortuitously, was continued as a matter of routine after it was found that acid phosphatase was not inhibited.

The tests were made by mixing equal volumes of the enzyme preparation, in 0·25 M-sucrose, and of the substrate which contained 0·16 M-*ABC* buffer and 0·1 M-sodium glycerophosphate adjusted to pH 5. The incubation time varied between 10 and 20 min. The reaction was stopped with 8% (w/v) trichloroacetic acid and the inorganic P determined on the filtrate according to Fiske & Subbarow (1925), using a Klett-Summerson photoelectric colorimeter. Suitable blanks were always run.

Preliminary assays performed with purified solutions of acid phosphatase showed that the reaction proceeded linearly with time, and that the activity was proportional to the amount of enzyme added, under the conditions of the tests.

RESULTS

Preliminary observations. The main observations which led to the present investigation are recorded in Tables 1 and 2. In this experiment, a complete fractionation of liver tissue was performed according to the technique outlined above, and tests for acid phosphatase carried out on the various fractions. The results of these tests (Table 1) show that a very poor activity balance sheet was obtained. Moreover, the activity of the initial homogenate was low, about one-tenth of the value usually observed in earlier experiments on aqueous liver extracts prepared in the Waring blender (Duve *et al.* 1949).

A second series of tests, made 5 days later on the fractions which had been kept at 2° showed that considerable activation of the preparations had occurred (Table 1). The aged homogenate exhibited a fairly 'normal' value, the recovery was more satisfactory and more than 50% of the total enzyme activity was found in the mitochondria fraction.

Table 1. *Cellular distribution of acid phosphatase activity in fresh and aged preparations*

(Fractionation of a 0·25 M-sucrose homogenate of rat liver.)

Fraction	Acid phosphatase activity* (mg. P/g. original tissue/10 min.)	
	Fresh preparations	After 5 days at 2°
Initial homogenate	0·16 (100)	1·34 (100)
Nuclei, washed twice	0·04 (25)	0·16 (12)
Mitochondria, washed twice	0·10 (63)	0·70 (52)
Microsomes, washed once	0·09 (56)	0·15 (11)
Final supernatant	0·09 (56)	0·14 (10)
Recovery	0·32 (200)	1·15 (85)

* Figures in brackets show activity distribution as percentage.

The same mitochondria were kept for 13 days at 2°, and then separated by centrifugation from the suspending fluid and washed once. Activity tests showed that only a small proportion of the enzyme had remained attached to the granules in the activated preparation (Table 2).

Two facts emerge from these experiments. In a fresh homogenate prepared in isotonic sucrose by the Potter-Elvehjem method, the acid phosphatase activity is low and is associated to a large extent with the mitochondria fraction; in an aged homogenate the activity is high and is no longer associated with the mitochondria.

Table 2. *Liberation of acid phosphatase from aged mitochondria*

(Mitochondria of Table 1, after 13 days at 2°, centrifuged 10 min. at 8500 *g* and washed once with 0·25 M-sucrose.)

Fraction	Acid phosphatase activity* (mg. P/g. original tissue/10 min.)
Aged mitochondria	0·73 (100)
Supernatant of first centrifugation	0·62 (85)
Precipitate of first centrifugation	0·11 (15)
Supernatant of second centrifugation (washing)	0·08 (11)
Precipitate of second centrifugation (washed)	0·03 (4)

* Figures in brackets show activity distribution as percentage.

It seemed likely that the low activity of the fresh preparation was a direct consequence of the bound state of the enzyme, and that 'activation' and 'liberation' were two related aspects of the same phenomenon. This conclusion has been substantiated by a large number of experiments, of which only representative examples will be described below.

Table 3. *Acid phosphatase activity after homogenization in the Waring blender*

(0·25 M-Sucrose homogenate of rat liver, prepared in the Waring blender (2 min.), centrifuged for 10 min. at 8500 *g*.)

Fraction	Acid phosphatase activity (mg. P/g. original tissue/10 min.)	
	Fresh preparations	After 2 days at 2°
Initial homogenate	1·45	1·70
Supernatant from centrifugation	1·50	1·60

Effect of the homogenizing device. When the Waring blender is used to homogenize the tissue, preparations of high activity are obtained, even in 0·25 M-sucrose or other isotonic solutions. In these preparations, the activity is present to a major extent in the supernatant after 10 min. centrifugation at 8500 *g*, and increases only slightly with ageing (Table 3). Experiments will be described below, showing that washed mitochondria exposed for 3 min. to the action of the Waring blender release their entire content of acid phosphatase.

These results explain the high activities observed earlier in water extracts prepared in the Waring blender (Duve *et al.* 1949). In these experiments, a second factor helped to release the enzyme from the mitochondrial complex, namely the distilled water used as homogenizing medium (see below).

The use of the Potter-Elvehjem homogenizer decreases to a large extent, but does not abolish completely the mechanical damage to the complex. The results of Table 4 indicate that a certain amount of enzyme, varying between 3 and 10 % of the total content of the mitochondria, is brought into solution with each homogenization. The quantities actually released are probably greater than those given in Table 4, since mitochondria adsorb a fair proportion of the liberated enzyme (Berthet, Berthet, Applemans & Duve, 1951).

Table 4. *Liberation of acid phosphatase by the Potter-Elvehjem homogenizer*

(Suspension of washed mitochondria submitted to three successive homogenizations, each of 2 min. duration. Before the experiment and after each run, a sample of the suspension is taken and centrifuged 10 min. at 20,000 *g*. Enzyme tests performed on the supernatants. The total activity, determined after exposure to the Waring blender, was 0·93 mg. P/g. original tissue/10 min.)

Number of homogenizations	Soluble acid phosphatase activity	
	mg. P/g. original tissue/10 min.	Percentage of total
0	0·038	4
1	0·063	7
2	0·10	11
3	0·20	21·5

Even the procedure used to resuspend the mitochondria after centrifugation must release a small quantity of enzyme, since suspensions of washed mitochondria always contain a little soluble activity.

Effect of the homogenizing medium. Various tests have been performed in order to find out in which homogenizing medium the highest proportion of bound enzyme can be recovered. Twin experiments were usually carried out, in which samples of the same liver pulp were homogenized in two different media. The two homogenates were then tested for their acid phosphatase activity, the lowest activity being taken as evidence that more of the enzyme was mitochondria linked. In some cases, the stability of the enzyme-mitochondria complex was also studied.

The following media were investigated: distilled water, 0·25 M-sucrose, 0·88 M-sucrose, 0·25 M-sucrose +7 % (w/v) serum albumin, 0·15 and 0·45 M-potassium chloride. Except for distilled water which causes a rapid liberation of the enzyme, all the above media furnish preparations of approximately

the same low activity. However, the data represented graphically in Fig. 1 show that spontaneous activation occurs more rapidly in isotonic potassium chloride than in isotonic sucrose.

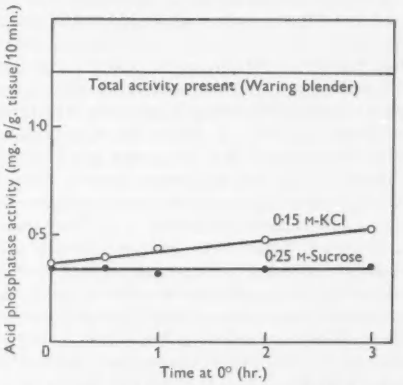

Fig. 1. Influence of KCl on spontaneous activation. Rat-liver pulp divided in two parts. Part 1 homogenized with 0·25 M-sucrose; part 2 with 0·15 M-KCl. The two homogenates kept at 0° and assayed regularly for acid phosphatase activity.

The best medium to obtain a high yield of stable mitochondria-linked acid phosphatase from rat liver appears to be salt free 0·25 M-sucrose. The 0·88 M-sucrose, advocated by Hogeboom, Schneider & Pallade (1948) for the preparation of intact mitochondria, has not proved more effective. Hogeboom & Schneider (1950) found that a change of 0·88–0·25 M-sucrose does not alter the distribution of total nitrogen, cytochrome *c* and a number of enzyme systems, and now recommend the latter medium for practical reasons.

Differential measurement of free and bound activity. The quantitative measurement of the total acid phosphatase content of a preparation offers no special difficulty, since a short treatment in the Waring blender produces complete activation. The data of Table 5 indicate that alternate freezing and thawing, if repeated at least six times, may be used for the same purpose.

On the other hand, in determining the free activity of preparations containing the enzyme partly in the bound form, precautions have to be taken against activation during assay. Various experiments were performed in order to assess the amount of activation occurring under different conditions.

In Fig. 2 are recorded the results of an experiment, in which washed intact mitochondria (*I*) and the same mitochondria, fully activated by a short preliminary exposure to the Waring blender (*B*), were incubated under two different conditions:

Table 5. *Activation by freezing and thawing*

(Preparations 1 and 3: mitochondria isolated from a 0·25 M-sucrose homogenate of rat liver, washed once. Preparation 2: precipitate after 1 hr. centrifugation at 20,000 *g* of a 0·25 M-sucrose homogenate of rat liver, containing nuclei, mitochondria and most of the microsomes, unwashed.)

Preparation no.	No. of successive freezings and thawings	Acid phosphatase activity (mg. P/g. original tissue/10 min.)
1	0	0·46
	1	0·70
	2	1·09
	3	1·18
2	0	0·16
	1	0·59
	2	0·78
	3	0·83
	4	0·82
	5	0·89
3	0	0·36
	6	1·15
	9	1·16
	12	1·20
	15	1·18
	16	1·19
Preparation 3 after 3 min. exposure to the Waring blender	0	1·16

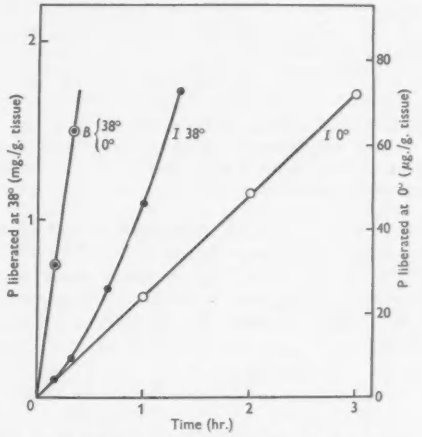

Fig. 2. Activation during the enzymic tests. *I*, washed mitochondria, intact; *B*, same preparation, treated 3 min. in the Waring blender. Incubation conditions: (*a*) 38°, pH 5, equal vol. of suspension in 0·25 M-sucrose and 0·16 M-*ABC* buffer + 0·1 M-glycerophosphate. (*b*) 0°, pH 6·1, equal vol. of suspension in 0·25 M-sucrose and 0·25 M-sucrose + 0·1 M-glycerophosphate.

(1) at 38° under the standard assay conditions; (2) at 0°, in 0·25 M-sucrose containing 0·05 M-glycerophosphate adjusted to pH 6·1. The course of liberation of inorganic P with time was followed.

As expected, the liberation of inorganic P is a linear function of time, both at 0° and at 38°, with the blender-treated preparation. It is 23·6 times slower at 0° than at 38° and the two curves may be made to coincide by using two different ordinate units bearing this ratio to each other. Plotted in the same manner, the curves for intact mitochondria coincide for the first 10 min., but subsequently diverge. The curve at 0° remains linear, indicating that no activation occurs in the test at low temperature. On the contrary, at 38°, progressive activation takes place as shown by the increasing rate of liberation of inorganic P.

The most important finding of this experiment is that the activation which occurs under the standard assay conditions does not begin immediately, but has a certain lag period. This lag period has been observed regularly and varies from 10 to 20 min.

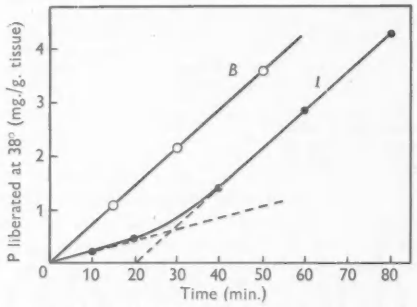

Fig. 3. Existence of lag period in activation at 38°. *I*, Washed mitochondria, intact; *B*, same preparation, treated 3 min. in the Waring blender. Standard incubation at 38° and pH 5.

It occurs even when the subsequent rate of activation is very great, as in the experiment represented in Fig. 3, in which full activation was reached between 20 and 40 min. incubation, the fastest rate of activation encountered so far in experiments of this type.

These experiments indicate that the true initial activity of preparations containing bound enzyme may be measured by the standard assay, provided that the incubation time does not exceed 10 min. An alternative method is to incubate at 0° and pH 6·1, in 0·25 M-sucrose containing 0·05 M- glycerophosphate.

Significance of the measured activities. The free activity observed with suspensions of washed mitochondria usually represents approximately 15 % of their total activity. A little less than half this amount is recovered in the supernatant fluid when the mitochondria are sedimented and is therefore due to fully active enzyme, set free in the process of resuspending the mitochondria. As will be shown in the following paper the residual activity which remains attached to the granules (8–10 % of the total) is due to a small amount of enzyme retained by non-specific and non-inhibitory adsorption to the surface of the mitochondria, the native bound form described in this paper having very little if any enzyme activity.

Consequently, the free activity of mitochondria, when determined under conditions which do not cause activation, is due essentially to the active enzyme present in the preparation, whereas the difference between the total and free activities must be considered a true measure of the amount of bound enzyme.

The quantitative distribution of acid phosphatase in the liver. In Table 6 are the results of a complete fractionation performed under what may be termed optimal conditions as the situation stands.

The technique described above was used, except for the following alterations. In order to minimize mechanical destruction of the mitochondria, these were washed only once; the supernatants from the centrifugations of the mitochondria were decanted very completely and subjected to an additional run of 10 min. at 8500 *g*, furnishing a small precipitate labelled 'intermediary fraction', and were then used for the separation of the microsomes.

Table 6. *The quantitative cytological distribution of acid phosphatase*

	Acid phosphatase activity					
	Fresh preparations (free enzyme)		Frozen preparations (total enzyme)		Difference (bound enzyme)	
Fraction	P*	%	P*	%	P*	%
Initial homogenate	265	17·1	1550	100	1285	82·9
Nuclei, washed twice	31	2	95	6·1	64	4·1
Mitochondria, washed once	87	5·6	950	61·3	863	55·7
Intermediary fraction, unwashed	33	2·1	190	12·2	157	10·1
Microsomes, washed twice	45	2·9	150	9·7	105	6·8
Final supernatant	134	8·6	163	10·5	29	1·9
Recovery	330	21·2	1548	99·8	1218	78·6

* μg. P/g. original tissue/10 min. All percentages calculated on the total activity, 1550 μg. P/g. tissue/10 min.

All the fractions were assayed for free enzyme activity by 10 min. tests, as soon as the fractionation was completed, and for total activity after freezing and thawing fifteen times.

Table 6 gives the amounts of free, total and (by difference) bound enzyme found in each fraction. The results show that the intial homogenate contains 83 % of bound enzyme and 17 % free. The distribution is slightly different when the data for the individual fractions is totalled, only 79 % of the enzyme being bound and 21 % free. These figures are entirely consistent with the observed damaging effects of the homogenizing and fractionation procedures, and strongly suggest that the whole of the acid phosphatase of liver is present in the intact cell in a mitochondria-linked form.

Of the complexes remaining intact 72 %, amounting to 56 % of the total activity, are recovered in the mitochondria fraction. A certain proportion is also found in other fractions, particularly in the intermediary and microsome fractions, which, in the Schneider procedure, would be combined into a single one.

As would be expected, a large part of the free activity is found in the final supernatant. The presence of free phosphatase in the other fractions is explained, partly by their content of fragile complexes, some of which are dissociated when the particles are resuspended, partly by their ability to adsorb the soluble enzyme.

Although only one complete experiment of this type has been performed, the distribution observed, in particular, the figure of 55–60 % for the recovery of acid phosphatase in the mitochondria, is fairly representative of the average of a large number of similar experiments. Thus in seven different homogenates of rat-liver tissue the mean total acid phosphatase activity, measured after exposure to the Waring blender or repeated freezing and thawing was $1{\cdot}52 \pm 0{\cdot}09$ mg. P/g. tissue/10 min., while the mean activity of sixteen preparations of mitochondria, in which somewhat less care was taken than in the foregoing experiment to recover the particles quantitatively, was $0{\cdot}82 \pm 0{\cdot}04$ mg. P/g. tissue/10 min., or 54 % of the mean total value.

A few qualitative experiments suggest that acid phosphatase is also mitochondria linked in the liver of rabbits and guinea pigs.

DISCUSSION

The results described above make it clear that a large part of the unspecific acid phosphatase of liver is linked to fairly large cytoplasmic granules in such a manner that the enzyme is inactive towards added glycerophosphate at pH 5. In all probability, the whole of the enzyme is particle bound in the intact cell.

The main questions raised by these findings are the nature of the linkage between enzyme and granule, the reason for the lack of activity of the bound enzyme and the nature of the phosphatase bearing granules. The two first questions will be dealt with more fully in the following paper. The third deserves some attention. From the general distribution of the bound enzyme, it would seem that the enzyme bearing particles belong to Claude's 'large granules', which have now been identified by numerous workers with the cell mitochondria. It should, however, be noted that a fairly large fraction of bound phosphatase fails to be recovered in the mitochondria fraction, but comes down with the microsomes and is even found to a small extent in the final supernatant. This fact, which comes out clearly in the results of Table 6, has been verified a number of times.

There are several possible interpretations for this observation, the simplest one being that a certain amount of sedimented mitochondria has been brought back into suspension, while decanting the supernatant after centrifugation. Although the possibility of some contamination occurring in this manner cannot be eliminated with certainty, it is doubtful whether it is the main factor responsible for the results obtained. In the first place, great care was taken in decanting the supernatant after centrifugation of the mitochondria and there was no indication that the packed precipitate had been disturbed in the course of this process. Neither did the supernatants contain any significant amount of granules visible at low magnifications. In addition, even after the supernatant has been subjected to an additional run of 10 min. at 8500 *g*, there is still found in suspension a significant amount (7 % of the total) of bound enzyme, which only sediments at 20,000 *g*, with the microsomes (Table 6).

Discounting the rather improbable possibility that mitochondria can be broken up into smaller fragments, while still retaining acid phosphatase in an inactive form, one is led to the conclusion that a given population of mitochondria may contain a significant proportion of smaller granules, which do not completely sediment in 10 min. at 8500 *g*. This, in turn, would appear to support the contention of Chantrenne (1947), who claimed on the basis of adenosinetriphosphatase and alkaline phosphatase measurements, that the distinction between mitochondria and microsomes is artificial and that a continuous spectrum of cytoplasmic granules exists, representing presumably various stages in the life cycle of these particles.

It is of particular interest in this connexion that liver tissue contains a second easily detectable enzyme, namely the specific glucose-6-phosphatase, which can be recovered to a very large extent in the microsome fraction, and which appears, from all the

evidence available, to be exclusively attached to submicroscopic granules (Hers *et al.* 1951). The distribution of this enzyme cannot be studied quite as accurately as that of acid phosphatase, owing to its lability and to the existence of interactions between the various fractions. However, the results, especially when they are corrected for the inactivation of the enzyme, which occurs mostly during the long centrifugations of the microsomes, provide a fair estimate of the distribution of glucose-6-phosphatase.

These measurements have been made on the fractions whose acid phosphatase activity is reported in Table 6 (Exp. 3 of Hers *et al.* 1951). It is therefore possible to establish in this particular experiment the content of each fraction of granules bearing acid phosphatase and glucose-6-phosphatase respectively. The distribution found is recorded in Table 7.

Table 7. *Distribution of acid-phosphatase- and glucose-6-phosphatase-bearing granules in fractions separated by centrifugation*

Fraction	Acid phosphatase-bearing granules (%)		Glucose-6-phosphatase-bearing granules* (%)	
Nuclei	4		6	
Mitochondria	56		13	
Intermediary	10	17	10	75
Microsomes	7		65	
Final supernatant	2		6	
Damaged	21		—	

* Corrected for inactivation of the enzyme.

The data of Table 7 make it clear that the distinction between microsomes and mitochondria is a valid one, but they also help to evaluate the limitations of the method of centrifugal fractionation in separating the two populations of granules. These limitations are not as apparent in other investigations in which the identification of the particles recovered in each fraction has rested mainly on chemical analyses and microscopic observations, and in which the distribution of more complex or less sharply characterized enzymic activities has been studied. In our experience the principal causes of error in a complete fractionation are the following.

Agglutination of particles with each other, and with particles of large size, causes them to come down in lower gravitational fields. As noted by other workers (Hogeboom *et al.* 1948; Kennedy & Lehninger, 1949; Hogeboom & Schneider, 1950), this phenomenon is enhanced by acidification, by an increase in ionic strength, and tends to increase with time. For instance, if the procedure for separating microsomes is shortened by centrifuging down the nuclei and the mitochondria simultaneously, as much as 95% of glucose-6-phosphatase can be recovered in the microsome fraction and in the final supernatant (Hers *et al.* 1951).

The relatively great variability in size of the particles in each population renders a certain amount of overlapping of the fractions inevitable. This appears to be true both for the microsomes and the mitochondria. In the former case, it may be estimated from glucose-6-phosphatase measurements that approximately 10% of the microsomes are too small to sediment completely in one hour at 20,000 *g* (Hers *et al.* 1951). The evidence presented here appears to show that an even larger proportion of the mitochondria is lost through incomplete sedimentation. It is our feeling that a better yield would be obtained by using a slightly higher gravitational field, such as 12,000–15,000 *g*, for the sedimentation of the mitochondria, for we have found that if only the upper half of the supernatant is collected after centrifuging 10 min. at 8500 *g*, practically no acid phosphatase bearing granules are recovered, an indication that even the lighter granules must already be well concentrated in the lower part of the tube at this stage.

The mitochondria are sensitive to mechanical damage. As will be shown in the following paper, the release of acid phosphatase is most probably the result of injuries to the mitochondrial membrane. The results presented in this paper show that such injuries are very easily sustained and that as much as 20% of the mitochondria may be damaged in this manner in the course of a fractionation experiment.

It is of course not possible to decide whether these conclusions are applicable to all cytoplasmic granules, since the general denomination of mitochondria or microsomes may cover a large number of populations of particles, similar in size and sedimentation rate, but differing in many other properties. That their applicability may not be restricted exclusively to the granules studied here is suggested by the fact that approximately 50% of another soluble protein which appears to be loosely bound to the mitochondria, namely cytochrome *c*, has been recovered, in the mitochondria fraction by Schneider & Hogeboom (1950). Indeed, the authors consider the possibility that all the cytochrome *c* may be mitochondria bound in the intact liver cell, a hypothesis which would appear to gain in probability from the results described in the present paper.

A study of the intracellular distribution of acid phosphatase in rat-liver cells has just been published by Palade (1951). The distribution found by this author is fairly similar to that described in this paper, except that he finds relatively less enzyme in the mitochondria fraction (40%), and relatively more in the other fractions. This difference can probably be explained by the fact that Palade's homogenates are prepared in hypertonic sucrose

(0·88 M) and are fractionated according to a scheme which is not directly comparable with ours. It is significant that in his experiments also, a fairly large proportion of the enzyme fails to sediment in gravitational fields sufficient to bring down all the large granules, and is partly recovered with the smaller granules.

A more important difference between Palade's work and ours lies in the conditions used for the enzyme assays. Palade's tests are performed in acetate buffer pH 4·5, with 0·08 M-β-glycerophosphate as substrate. Enzyme and substrate are first equilibrated separately for 10 min. at 38° in the two parts of a Warburg vessel, mixed and incubated for 1 hr. with continuous shaking. These conditions favour activation during the assay and probably explain why Palade did not observe the low activity of the bound enzyme or the various types of activation described in this paper.

As pointed out above, these features have an important bearing on the final interpretation of the results of distribution experiments.

SUMMARY

1. In fresh rat-liver homogenates prepared in 0·25 M-sucrose, a large proportion of the acid phosphatase is linked with the mitochondria.

2. The bound enzyme has little or no activity towards added glycerophosphate at pH 5.

3. Ageing of the preparations at 0°, short exposure to the Waring blender, repeated freezing and thawing, all lead to an irreversible release of the enzyme, which then appears to a large extent in a soluble form. Partial damage to the complex is caused by the procedures used to homogenize and fractionate liver tissue.

4. In contrast to its bound form, the free enzyme exhibits a high activity.

5. When mitochondria are separated by differential centrifugation and washed once, they are found to contain 55–60 % of the total acid phosphatase content of the original homogenate. The remainder is found partly in the smaller granules, partly in the final supernatant. The nuclear fraction contains only a low percentage of the total activity.

6. Analysis of these results indicates: (*a*) that the whole acid phosphatase content of rat liver must be particle-bound in the intact tissue; (*b*) that the ability to bind acid phosphatase in the manner described belongs to a population of granules which are fairly heterogeneous in size. About one-third of them are too small to sediment completely in 10 min. at 8500 *g*.

7. The implications of these findings, in particular with respect to the technique of centrifugal fractionation, are discussed.

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Tissue Fractionation Studies

2. THE NATURE OF THE LINKAGE BETWEEN ACID PHOSPHATASE AND MITOCHONDRIA IN RAT-LIVER TISSUE

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In the preceding paper (Berthet & Duve, 1951), the existence in rat-liver tissue of a mitochondria-linked form of acid phosphatase has been described and the cytological significance of this finding has been discussed. The nature of the linkage between enzyme and granule forms the object of the present communication.

Two facts relevant to this problem have already been reported, namely the great lability of the complex, which can be disrupted quantitatively by mechanical or physical means (Waring blender, freezing and thawing, hypotonic media), and its lack of enzymic activity towards added glycerophosphate at pH 5. Considerations based on these and other properties to be described in this paper, indicate that the enzyme is retained inside the mitochondrial structure by a membrane-like barrier, endowed with selective permeability and impermeable to glycerophosphate. A preliminary report of this work has already been published (Duve, Berthet, Berthet & Appelmans, 1951).

METHODS

The following general procedure has been followed in many of the experiments which will be reported. Mitochondria were separated from rat-liver tissue by the method previously described (Berthet & Duve, 1951), washed once and resuspended in a small quantity of cold 0·25 M-sucrose. Samples, of this suspension were suitably diluted with media of given composition and incubated. Except in the cases where the effect of temperature was studied, the incubation was carried out in the cold room, in an ice-water bath.

At various times after the beginning of the experiment, samples of the diluted suspension were centrifuged 10 min. at 20,000 *g*. Part of the supernatant was collected and kept for enzyme assay. A sample for the determination of the starting value was obtained in a similar way from a 0·25 M-sucrose dilution of the mitochondria. Another portion of the mitochondria was treated for 3 min. in the Waring blender, for the measurement of the total enzyme content.

When all the samples had been collected, their acid phosphatase activity was determined according to the technique described previously to provide an index of the amount of soluble enzyme present at the time the suspension was centrifuged.

In a number of experiments, additions to this procedure or deviations from it were introduced; these are mentioned in the text.

RESULTS

Effect of temperature. When mitochondria are kept in 0·25 M-sucrose at 0°, acid phosphatase is released very slowly, as shown by many of the graphs reproduced below. Raising the temperature produces a marked increase in the rate of liberation of the enzyme, and the activation which occurs under those conditions follows a characteristic course. The results which are represented graphically in Figs. 1–3 illustrate various aspects of this phenomenon.

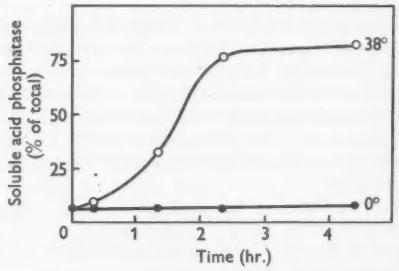

Fig. 1. Influence of temperature on release of acid phosphatase in 0·25 M-sucrose.

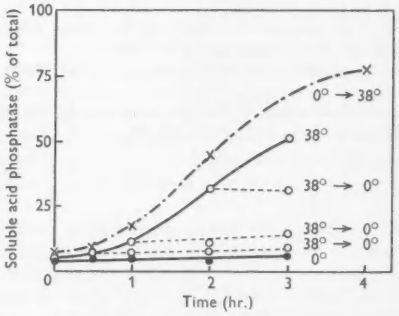

Fig. 2. Influence of pre-incubation at 38 or 0° on subsequent release of acid phosphatase at 0 and 38° respectively in 0·25 M-sucrose. ●——●. Fresh mitochondria, 0°; ○——○, fresh mitochondria, 38°; ○- - -○, brought back to 0° after preincubation at 38°; ×—·—×, 38°, after 3 hr. at 0°.

An experiment in which full activation was obtained after approximately 2·5 hr. incubation at 38° is shown in Fig. 1.

The results of a similar experiment, in which, in addition, the effects of pre-incubation at 38 and 0° on the subsequent rate of release at 0 and 38° respectively were investigated, are given in Fig. 2.

Fig. 3 shows in diagrammatic form the amounts of soluble acid phosphatase present in a 0·25M-sucrose suspension of mitochondria before and after standing 2·5 hr. at various temperatures.

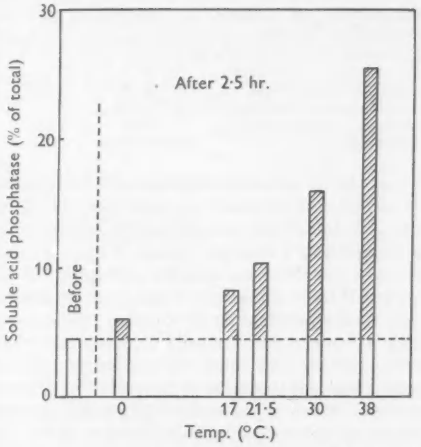

Fig. 3. Percentage of acid phosphatase released after 2·5 hr. standing at various temperatures in 0·25M-sucrose.

The main conclusion which may be drawn from these experiments is that chemical reactions are involved in the activation. This is supported by the magnitude of the temperature effect and by the general aspect of the results of Fig. 3. Although the particular shape of the activation curve does not allow a more detailed analysis of these results, it may further be deduced from Fig. 2 that liberation of the enzyme must be intimately linked with a temperature-sensitive reaction or reactions, since a previous incubation at 38° does not increase the rate of release at 0°. On the other hand, some change must occur even at 0°, in view of the increased rate of thermal activation which takes place after 3 hr. standing at this temperature.

Another significant point is the characteristic S-shape of the activation curve shown in Fig. 1. The slow initial rate of release of the enzyme and its progressive increase with time, are reminiscent of similar features which have already been described (Berthet & Duve, 1951), for the activation which occurs when mitochondria are incubated at 38° in a mixture containing equal volumes of 0·25M-sucrose and of the substrate used for the enzyme assays. The reason for this similarity will be given below. The abrupt flattening of the curve at a point where only 80% of the enzyme has dissolved is explained by the fact, discussed in the last section, that a fraction of the enzyme always remains attached to the particles, even in fully active preparations.

Effect of distilled water. Dilution of 0·25M-sucrose suspensions of mitochondria with 15–25 vol. of distilled water causes a very rapid activation, even at 0°. The results of seven experiments of this

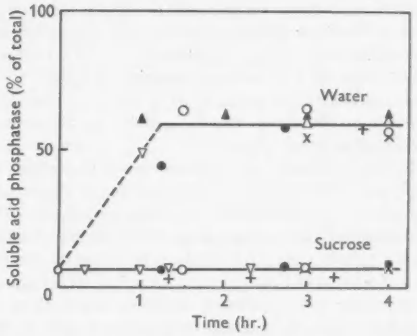

Fig. 4. Effect of distilled water on release of acid phosphatase. The signs represent independent experiments.

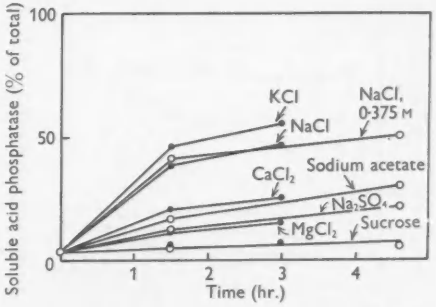

Fig. 5. Effect of ionic composition of suspending medium on release of acid phosphatase. All concentrations, 0·25M, except NaCl where indicated in graph.

type have been condensed in Fig. 4, which shows that a plateau corresponding to the release in soluble form of approximately 60% of the total enzyme content, is reached at the end of 1 hr. Here again, as will be shown below, the preparation attains maximum activity, even though a considerable part of the enzyme remains attached to the particles. Moreover, other observations suggest that the actual activation phenomenon occurs very

rapidly and is not preceded by a lag period as observed for thermal activation.

Effect of salts. The action of salts is characteristic. In the absence of sucrose, they are unable to prevent a progressive liberation of the enzyme, even when present in hypertonic concentration. This effect is particularly rapid with potassium chloride and sodium chloride; it is much slower with sodium acetate, calcium chloride, magnesium chloride and sodium sulphate (Fig. 5).

In order to avoid any error in the estimation of soluble enzyme in the experiments described in Fig. 5, parallel tests were made in the presence of equivalent concentrations of the various salts used, with a dialysed preparation of acid phosphatase, extracted from mitochondria with distilled water. Inhibitions of 5 % with potassium chloride, 20 % with calcium chloride and 38 % with sodium sulphate were observed, and the results obtained were corrected accordingly.

In the presence of 0·25 M-sucrose, the effect of salts is entirely different. Except for a slight and practically instantaneous increase in soluble acid phosphatase, the significance of which will be discussed below, they exert little or no influence on the rate of release of the enzyme. A typical experiment, illustrating the difference between the actions of 0·44 M-sodium chloride in the presence and in the absence of 0·25 M-sucrose is represented in Fig. 6.

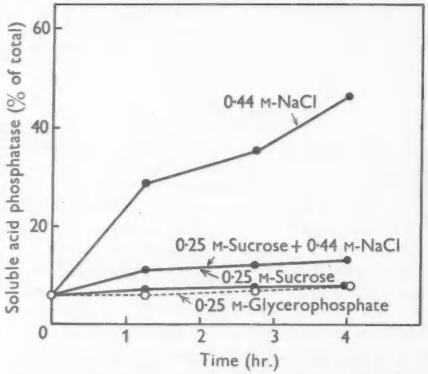

Fig. 6. Inhibition by 0·25 M-sucrose of activating effect of 0·44 M-NaCl. Protection by 0·25 M-glycerophosphate.

Additional information concerning the mechanism by which salts effect liberation is furnished in Table 1. In this experiment, mitochondria were suspended in 0·25 M-sucrose containing 0·5 M-sodium chloride and centrifuged. The supernatant contained 12·5 % of the total activity. Equal quantities of the sedimented granules were resuspended in 0·25 M-sucrose + 0·5 M-sodium chloride and in 0·25 M-sucrose respectively, and assayed for free enzyme activity. Whereas the activity of the salt containing suspension was only 23 % of the total, the granules resuspended in sucrose alone possessed 89 % of the total activity and had therefore become fully active.

Table 1. *Disruption of salt-enriched mitochondria in 0·25 M-sucrose*

Preparation	Acid phosphatase activity (mg. P/g./10 min.)	Acid phosphatase activity (% of total)
Total activity of original mitochondria (blender)	0·71	100
Supernatant of washing with 0·25 M-sucrose + 0·5 M-NaCl	0·09	12·5
Precipitate, resuspended in 0·25 M-sucrose + 0·5 M-NaCl	0·12	23
Precipitate, resuspended in 0·25 M-sucrose	0·63	89

These results are easily explained if it be admitted that mitochondria behave as osmotic systems, impermeable to sucrose but permeable to water and various salts, and that the release of the enzyme is the consequence of an osmotic disruption of the granules. When suspended in water, their breakdown is due to the penetration of water, a phenomenon which is very rapid. In salt solutions the same process occurs, but only when enough salt has diffused into the granules to decrease the effective osmotic pressure of the surrounding medium below the critical value. The rate of diffusion of the salt thus becomes the limiting factor. In the presence of 0·25 M-sucrose, osmotic disruption is prevented, but not the penetration of salt. Consequently, such salt-enriched mitochondria will disintegrate if they are resuspended in pure sucrose, because water enters the granules more rapidly than the salt can leave them (Table 1).

In addition to these main effects, there are other secondary actions of salts which will be discussed in the last section.

Effect of glycerophosphate and of the enzyme substrate. In several experiments, a solution of 0·25 M-sodium glycerophosphate adjusted to pH 6·1 with hydrochloric acid was used as suspending medium. In all these experiments, the curve of release of the enzyme was found to coincide perfectly with that observed in 0·25 M-sucrose. An example is given in Fig. 6. In terms of the osmotic interpretation put forward above, this implies that mitochondria are as impermeable to glycerophosphate as to sucrose, a fact sufficient to account for the lack of detectable activity of the bound enzyme.

A similar test was carried out using as suspending medium equal vol. of 0·25 M-sucrose and of the enzyme substrate. This duplicated the conditions under which the assays on intact mitochondria, described in the preceding paper (Berthet & Duve,

1951), were performed. A very slow liberation occurs in the medium, so that it is almost as effective as 0·25M-sucrose in protecting the granules against osmotic disruption. The fact that little activation takes place during the first 10 min. of the enzyme assays—an important factor in the discussion of the results reported in the preceding paper—is thus entirely explained, since the initially slow thermal activation becomes dominant in the absence of osmotic factors.

The protection afforded by this medium is not surprising when its composition, which is given in Table 2, is considered. The sucrose and glycerophosphate concentrations alone, make the medium

Table 2. *Composition of incubation medium for standard enzyme assay*

Component	Molarity
Sucrose	0·125
Glycerophosphate (8% doubly ionized)	0·05
Boric acid	0·08
Cacodylic acid (8% ionized)	0·08
Acetic acid (70% ionized)	0·08
Na^+	0·30
Cl^-	0·185
Total	0·900

iso-osmotic with 0·175M-sucrose. If the mitochondria are only slightly permeable to boric or cacodylic acid, the effective osmotic pressure should reach a value equivalent to that of 0·25M-sucrose. It is quite possible that the medium is entirely adequate osmotically, and that the small activating effect it produces is due to its low pH (pH 5).

As pointed out previously (Berthet & Duve, 1951) the use of the *ABC* buffer mixture was fortuitous, since acetate alone would have been just as effective as a buffer. However, it appears that the other components of the buffer served an unexpected purpose. The use of this buffer and the adoption of short incubation periods, made it possible to demonstrate the lack of activity of the bound enzyme, a property which can become completely obscured when other assay conditions are used, as in the experiments of Palade (1951).

Effect of other substances and of detergents. Fig. 7 illustrates the behaviour of the acid phosphatase-bearing mitochondria in 0·25M-glucose and 0·25M-glycerol. Glucose is not as effective as sucrose in protecting the granules and probably enters them at a slow but significant rate. With glycerol, activation is very rapid. As in previous similar cases, the amount of soluble enzyme present reaches a plateau far below the total content of the granules.

The action of two detergents, saponin and sodium deoxycholate, was also investigated. In these, as in some of the experiments with salts, the results are complicated by inhibiting effects. These were measured separately on a preparation of soluble acid phosphatase. In Fig. 8 are represented the quantities of soluble enzyme present in 0·25M-sucrose suspensions of mitochondria kept for 1 hr. at 0° with various concentrations of the two detergents, corrected for inhibition effects. It is of interest to note that approximately one-tenth of the concentration of both saponin and deoxycholate necessary to produce full activation of the mitochondria is sufficient to cause complete haemolysis of human erythrocytes.

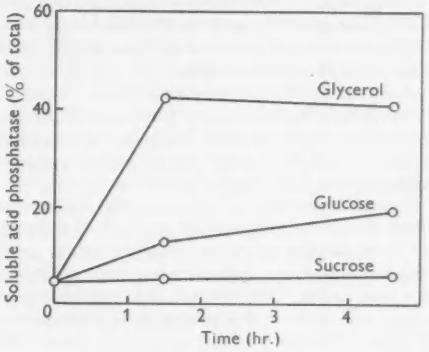

Fig. 7. Effect of 0·25M-glucose and glycerol on release of acid phosphatase.

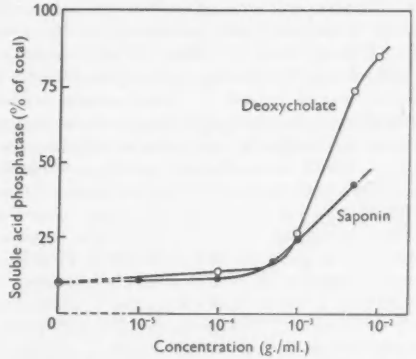

Fig. 8. Acid phosphatase released after 1 hr. standing in the presence of various concentrations of saponin or sodium deoxycholate.

Effect of pH. This was only studied in media which were made isotonic partly with sucrose, partly with salts, so that the results are complicated by osmotic effects. In an experiment covering the pH range from 3·7 to 8·5, maximum stability was observed between pH 5·5 and 6·5, with a slight and progressive increase in the rate of activation at values below or above this limit.

Retention of active enzyme by particulate material. It will have been noticed that in all the experiments described above, in which the activation has reached a limiting value, the amount of soluble enzyme present in the mitochondria suspension is always less than the total acid phosphatase content. This is true whatever the activating agent used, whether it be an increase in temperature (Fig. 1), distilled water (Fig. 4) or 0·25 M-glycerol (Fig. 7). It is also true in the case of mitochondria treated in the Waring blender or disrupted by freezing and thawing. Only in one instance, with mitochondria which had aged 13 days at 0° (Berthet & Duve, 1951), was almost the entire enzyme content of the granules in the soluble form.

Another aspect of this phenomenon, illustrated by the data in Table 3, is that the amount of enzyme retained by the particulate material varies with the nature of the activating agent, and is relatively constant with each type of activating agent.

Finally, there are two experimental observations which have a bearing on this problem, namely that the free activity of intact mitochondria is always larger than their non-sedimentable activity (Berthet & Duve, 1951), and the peculiar liberation produced by salts in the presence of 0·25 M-sucrose (Fig. 6).

A point of special importance in understanding these phenomena appeared to be the actual activity of the enzyme retained by the particulate material in all these experiments. In order to investigate this matter, tests were first performed on the resuspended precipitates, but these did not furnish unequivocal results, because it is impossible to resuspend a precipitate of mitochondria without causing some mechanical damage. Moreover, this damage is greater when the mitochondria have been treated with a salt-containing solution of sucrose, even if the same medium is used to resuspend them. Salts, in addition to the effects already described, cause a loosening of the mitochondrial structure. This effect is generally not sufficient to produce an actual liberation of bound acid phosphatase, but renders the granules more sensitive towards other activating agents. It is perhaps related to the fact, demonstrated by spectrophotometric determina-

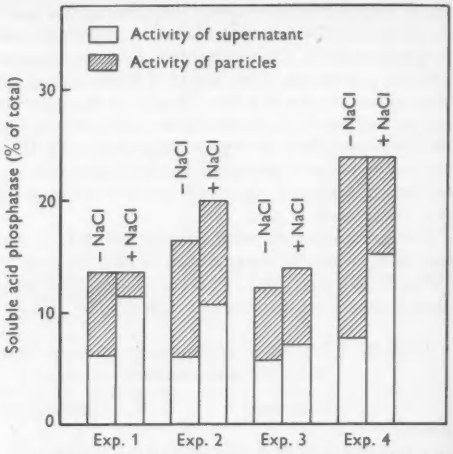

Fig. 9. Effect of 0·15 M-NaCl on distribution of free acid phosphatase activity between supernatant and particles in intact mitochondria.

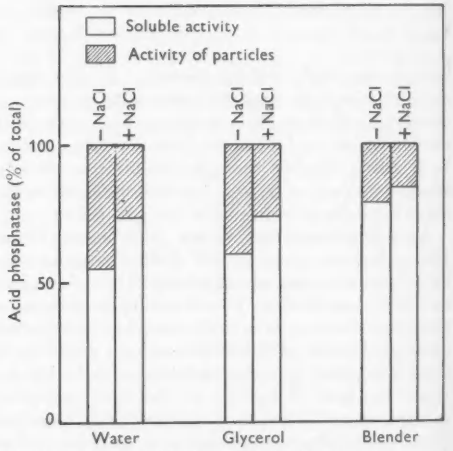

Fig. 10. Effect of 0·15 M-NaCl on distribution of free acid phosphatase activity between supernatant and particles in disintegrated mitochondria.

Table 3. *Retention of acid phosphatase by particulate material after full activation*

Activating agent	No. of exp.	Acid phosphatase retained by particulate material, % of total: Unwashed precipitate	Precipitate washed once	Precipitate washed twice
Waring blender	5	20±2	—	—
Distilled water	6	40·6±1·4	—	—
Freezing and thawing	1	32	19	19
Incubation at 38° in 0·25 M-sucrose	1	20	—	—
0·25 M-glycerol	2	59	—	—
		39		
Ageing at 0° (13 days)	1	15	4	—

Table 4. *Adsorption of acid phosphatase by intact mitochondria*

Suspension medium	Acid phosphatase (μg. P/10 min.) Supernatant of mitochondria	Soluble enzyme added	Supernatant of mitochondria + added enzyme	Difference (adsorbed enzyme)
0·25 M-sucrose	5·8	66	54·1	17·7
0·25 M-sucrose + 0·15 M-NaCl	13·1	66	73·7	5·4

tions, that nucleic acids are detached, apparently from the surface of the mitochondria, under the influence of strong salt solutions, even in 0·25 M-sucrose.

In order to circumvent these difficulties, tests were made on the original suspensions and on the supernatants, the differences between the two values being taken as representing the activities of the particles. Special precautions were taken to prevent activation during the tests, which were carried out at either 38 or 0°, under the conditions described in the preceding paper for intact mitochondria (Berthet & Duve, 1951). Fig. 9 shows diagrammatically the results of four experiments of this type, with intact mitochondria suspended in 0·25 M-sucrose or in 0·25 M-sucrose + 0·15 M-sodium chloride. It is clear that the main effect of the salt is to detach from the granules a fraction of enzyme which is already fully active when bound. In two experiments, a slight activation is also observed, a consequence of the labilizing effect of salts, described above.

In Fig. 10 are the results of a similar experiment on mitochondria which had been activated by distilled water, 0·25 M-glycerol and treatment in the Waring blender respectively. They show unambiguously that the portion of enzyme retained by the particles is fully active in all cases and that it is partly brought into solution, as in the experiments on intact mitochondria, by the addition of 0·15 M-sodium chloride.

Table 5. *Adsorption of acid phosphatase by microsomes*

Fraction	Acid phosphatase activity (μg. P/10 min.)
Washed microsomes	4
Microsomes + acid phosphatase	101·2
Supernatant of first centrifugation	89·2
Precipitate of first centrifugation	11·2
Supernatant of second centrifugation	2·7
Precipitate of second centrifugation	9·0
Supernatant of third centrifugation	0·6
Precipitate of third centrifugation	6·2

From these results it may be concluded that the linkage by which a portion of acid phosphatase remains attached both to intact and disintegrated mitochondria, does not modify the activity of the enzyme, and must therefore be different from that in the native complex which is entirely inactive.

It is probable that several different phenomena are responsible for this second type of linkage. There is no doubt that part of the enzyme is retained by adsorption, and that salts have an eluting effect on this adsorption complex. The data of Table 4 refer to an experiment, in which adsorption of dissolved acid phosphatase by intact mitochondria, and its partial elution by sodium chloride, could actually be observed. As shown by Table 5 and by observations described in the preceding paper (Berthet & Duve, 1951), the ability to adsorb the enzyme is not restricted to mitochondria, but is shared by microsomes and by nuclei. It may therefore be considered a non-specific phenomenon. A peculiar fact is that 0·25 M-sodium-glycerophosphate, adjusted to pH 6·1 with hydrochloric acid, causes no elution, although it contains a fair amount of sodium chloride (Fig. 6).

Mitochondria which have been disrupted osmotically (water, glycerol), may in addition to the adsorbed fraction, retain another fraction of enzyme trapped inside the collapsed granule. That some such phenomenon may take place is suggested by the results of Table 6, which show that an additional quantity of enzyme can be extracted with water from a precipitate of mitochondria disrupted with distilled water and centrifuged down in the presence of sodium chloride.

Table 6. *Extraction of acid phosphatase with distilled water from mitochondria disrupted in distilled water and washed with salt*

Fraction	Soluble acid phosphatase (% of total)
Original suspension	4
A. In supernatant, after 4 hr. in distilled water	62
B. In supernatant of A, with previous addition of NaCl to 0·15 M concentration	80
C. In supernatant of precipitate from B, resuspended in distilled water	16

Finally, one cannot exclude the possibility that a small portion of the enzyme is firmly attached to sub-microscopic granules forming part of the mitochondrial inclusions.

DISCUSSION

In view of the combined evidence presented in this and the preceding paper, it may be safely concluded that the linkage between acid phosphatase and its particulate support cannot be a simple molecular association, either chemical or physical; obviously structural factors are involved. The information, obtained by methods which are entirely self-sufficient, requires no predefined morphological picture for its interpretation.

The evidence presented indicates that the enzyme acid phosphatase is associated, in a manner which suppresses its activity under the usual assay conditions, with a group of fairly large cytoplasmic entities, of which two-thirds are sedimented in a 0·25 M-sucrose homogenate of liver tissue, by centrifuging for 10 min. at 8500 *g*. The enzyme can then be extracted in fully active form from these entities by a number of methods.

Of the various agents which are found to bring about partial or total release of the enzyme, there are several which may be termed unspecific, in that they do not permit any definite conclusion to be drawn as to the structure of the carrier granule. For instance, the fact that exposure to the Waring blender or to repeated freezing and thawing causes liberation of acid phosphatase, simply shows that the binding of the enzyme requires a certain structural integrity of the carrier granule, but does not help to characterize further the nature of its structure.

There is, however, a group of facts which do not admit of several interpretations. The enzyme is only retained by its carrier if a certain kind of substance, which may be ionic (glycerophosphate) or non-ionic (sucrose) in nature, is present in sufficient concentration in the suspending medium. A progressive release occurs when these substances are replaced, in equimolar concentration, by a variety of others, which may also be ionic (sodium chloride, potassium chloride) or not (glycerol). The rate at which this phenomenon takes place depends on the nature of the substance present. The addition of a protecting substance in adequate concentration is sufficient to suppress completely the liberation which occurs in the presence of non-protecting substances. These facts can only be understood if it be admitted that the enzyme-bearing granules behave as osmotic systems.

Consequently, any representation of these granules must at least include the essential features of an osmotic system: a limiting barrier capable of depressing to a greater or lesser extent the diffusion of solutes present within or outside the granule; an interior medium containing a number of osmotically active components, to which the barrier is not permeable. The simplest model obeying this description is that of a sac, consisting of fluid enclosed in a semi-permeable membrane.

All the facts reported in this work can be fitted into this picture, simply by assuming that acid phosphatase is present in a diffusible form inside the sac. Mechanical agents, such as the Waring blender and other homogenizing devices, may cause injuries to the membrane. Media which are hypotonic with respect to osmotically effective substances, to which the membrane is impermeable, will induce swelling of the granules, with the consequent distention or disruption of the membrane. Detergents are known to dissociate lipoprotein complexes and to disintegrate biological membranes. Repeated freezing and thawing probably acts in several ways, by splitting or denaturing architectural components, creating local increases in osmotic pressure and causing tearing effects due to ice crystals. Finally, the thermal activation may be interpreted as a consequence of autolytic changes affecting the membrane, and the particular S-shape of the curve might indicate either that these changes are autocatalytic or that the 'survival-time' of the granules is dependent on a statistically distributed property. In all cases, therefore, the release of acid phosphatase may be related to damages suffered by a membrane. In addition, the impermeability of the membrane to glycerophosphate, which is demonstrated by the protection afforded by this substance, is sufficient to explain the lack of detectable enzymic activity when intact granules are incubated under mild conditions with glycerophosphate.

This picture is complicated to a certain extent by side phenomena, most of which are due to non-specific secondary adsorption of the enzyme on particulate material.

It may be concluded that a sac-like representation of the acid phosphatase-bearing granules is both necessary and sufficient to account for their experimentally determined behaviour, at least as far as the release or the retention of acid phosphatase is concerned. This does not mean, of course, that the content of the sac is not itself structurally organized. On the contrary, it is very probable that an internal structure exists, but its study is beyond the scope of the methods used in this work.

There is one alternative interpretation which, though not very probable, should be mentioned, namely that the membrane does not surround the whole granule, but only that part of it containing acid phosphatase.

A second point, which has already been discussed (Berthet & Duve, 1951) concerns the nature of the granules to which these conclusions apply. We do not know what these granules are, and it is only because they appear largely in a fraction whose content has been identified by several workers with the cell mitochondria that we have used this name to

characterize them. At least, if they are mitochondria, then in the cytoplasm of liver cells a significant amount of smaller mitochondria must exist which do not sediment completely in 10 min. at 8500 *g*, since the acid phosphatase-bearing particles which are recovered in the microsome fraction show all the properties of the larger ones, and it is reasonable to assume that both groups are part of a homogeneous population of granules of varying size.

There is very good agreement between the conclusions reached in this work and the results which have been obtained by other methods. Both Claude (1946) and Hogeboom, Schneider & Pallade (1948) state that large granules or mitochondria isolated by differential centrifugation swell and disrupt in hypotonic media, and consider this phenomenon to be osmotic in nature. It is also mentioned by Hogeboom *et al.* (1948) that mitochondria prepared in saline are less stable than in sucrose. The existence of a structurally defined membrane around the mitochondria has been further demonstrated by electron microscope studies (Claude & Fullam, 1945; Dalton, Kahler, Kelly, Lloyd & Striebich, 1949). The observations of the latter authors are of special interest, describing various stages in the disruption of mitochondria, from incipient leakages of the mitochondrial contents up to completely empty 'ghosts', occurring in isotonic saline.

The membrane theory has been disputed by Huennekens & Green (1950) on the basis of results showing that some pyridino-proteins of the 'cyclophorase-complex' can be selectively dissociated into apoenzyme and coenzyme. However, their data do not disprove the existence of a membrane, but show simply that the coenzymes cannot be considered as simply in solution in the intramitochondrial fluid. In addition, their results, like those of Harman (1950), which have also been quoted against the membrane hypothesis, are obtained on material separated by low-speed centrifugation from homogenates prepared in 0·9% potassium chloride by means of the Waring blender. Both these factors have been shown to cause considerable damage to the mitochondrial structure.

SUMMARY

1. The influence of various physical and chemical agents on the ability of cytoplasmic granules to retain acid phosphatase has been investigated.

2. The best protection of the complex is observed when the granules are kept at 0° in a medium containing 0·25 M-sucrose or glycerophosphate.

3. When these substances are absent or replaced by a variety of others, a progressive release of the enzyme occurs, at a rate which is dependent on the nature of the substance added. The phenomenon is very rapid in distilled water, 0·25 M-glycerol, and 0·15 or 0·44 M-sodium chloride or potassium chloride. It is completely inhibited when 0·25 M-sucrose is present as well in the suspending medium.

4. The rate of release in 0·25 M-sucrose is increased by an increase in temperature, and by addition of small amounts of saponin or deoxycholate.

5. Acid phosphatase is easily adsorbed by various structural entities. In contrast to the native bound form of the enzyme, the adsorbed form is fully active enzymically, and is partly eluted by salts.

6. From a consideration of all the experimental data, it is concluded that the acid phosphatase-bearing granules are osmotic systems and must therefore possess a semi-permeable membrane and an osmotically active internal medium.

The authors wish to thank the organisations which have contributed to the cost of this work, namely the 'Lilly Research Laboratories', the 'Fonds National de la Recherche Scientifique' and the 'Centre National de Recherches sur la croissance normale et pathologique'.

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The Function of Carbonic Anhydrase in the Stomach

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Berend (1937) suggested that part of the mechanism of gastric hydrochloric acid production might be the catalysed hydration of carbon dioxide to carbonic acid by carbonic anhydrase, but he failed to find significant quantities of the enzyme in the stomach. However, Davenport & Fisher (1938) showed conclusively that there are large amounts in gastric mucosa, and Davenport (1939, 1940*a*) found that most of the gastric carbonic anhydrase is in the oxyntic cells.

Davenport (1940*b*) discovered that thiocyanate could inhibit both carbonic anhydrase activity and acid secretion in dogs, but Feldberg, Keilin & Mann (1940, 1948) found that concentrations of thiocyanate, which strongly inhibited gastric acid secretion in decerebrate cats, inhibited gastric carbonic anhydrase by only about 10%. They also found that concentrations of sulphanilamide sufficient to inhibit the enzyme between 80% and 'completely' did not decrease the rate of acid secretion. Similar failures to inhibit acid secretion in dogs, cats or turtles with sulphanilamide or the more powerful inhibitor, thiophene-2-sulphonamide were reported by Davenport (1941, 1946*a*) and by Anderson & Wilbur (1948).

Since there is an uptake of one molecule of carbon dioxide to produce a bicarbonate ion for each hydrogen ion secreted, hydration of carbon dioxide certainly occurs in oxyntic cells (Davies, 1948). Moreover, Davies & Roughton (1948) have calculated that (*a*) this hydration is so fast that carbonic anhydrase activity is needed within mammalian and amphibian oxyntic cells, and (*b*) the amount of enzyme activity in the cells is apparently in great excess of the requirements. Only about 0·3% or less of the enzyme activity extractable from oxyntic cells is actually required to catalyse the uptake of carbon dioxide even during high rates of hydrochloric acid secretion.

The failure of previous attempts to inhibit acid secretion by inhibitors of carbonic anhydrase can be explained as follows. Roughton (1943) has shown that complete inhibition of this enzyme in the blood should lead to the speedy death of the animal. Since all the animals used in these experiments were respiring normally, there must have been sufficient residual carbonic anhydrase activity in the red cells and, since there is about five times as much of this enzyme in oxyntic cells as in red cells (Davenport, 1939), the gastric enzyme could not have been completely inhibited *in vivo*.

This difficulty has been avoided in the experiments described below by the use *in vitro* of tied bags of isolated frog and toad gastric mucosa. After virtually complete inhibition of the carbonic anhydrase, acid secretion ceased and the mucosa was damaged. Such damage did not occur in similar experiments with non-secreting gastric mucosa.

A part of the present work was communicated to the Biochemical Society (Davies & Edelman, 1948).

EXPERIMENTAL

Methods. The saline media, manometric procedure, treatment of tissues and methods of estimation of the rates of acid secretion were those described by Davies (1948) and Davies & Longmuir (1948).

The muscle layers were removed from the well washed stomach of a frog or toad, and the tube of gastric mucosa formed was tied with silk at both ends. The tied bag was incubated in a glucose-containing physiological saline solution in a double-armed Warburg cup containing histamine (final concentration 5×10^{-5}M) in one arm, and a solution of the inhibitor in the other. Acid secretion commenced either spontaneously or following the addition of histamine, and the inhibitor was then added to see if it interfered with the normal course of secretion.

All experiments were performed at least in triplicate, and usually in quintuplicate. Concordant results were always obtained. Values of Q_{O_2} and Q_{HCl} are in μl./mg. dry wt./hr.

Inhibitors. The carbonic anhydrase inhibitors used were sulphanilamide (Mann & Keilin, 1940), thiophene-2-sulphonamide (Davenport, 1945), *p*-toluenesulphonamide, *p*-sulphonamidobenzoic acid and 4′-sulphonamidophenyl-2-azo-7-acetamido-1-hydroxynaphthalene-3:6-disulphonic acid (prontosil soluble) (Krebs, 1948). They were all neutralized with NaOH before use.

Estimation of carbonic anhydrase activity. At the end of the incubation the gastric mucosa was ground with sand and the carbonic anhydrase activity of aqueous extracts estimated at 0° by the manometric method of Krebs & Roughton (1948). Sufficient inhibitor was added to the phosphate and bicarbonate solutions used in this method to keep the concentration the same as that in the medium during the original incubation of the mucosa.

RESULTS

Effects on acid secretion. Preliminary experiments showed, as anticipated, that a relatively high concentration of carbonic anhydrase inhibitor was needed to affect acid secretion by the oxyntic cells.

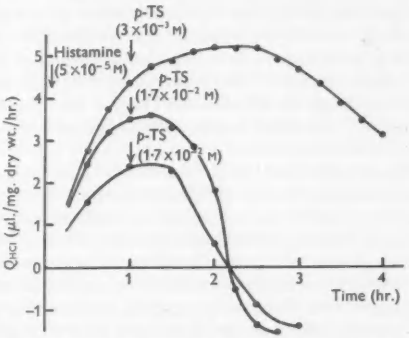

Fig. 1. Effect of *p*-toluenesulphonamide on acid secretion by isolated frog gastric mucosa. Histamine added first, followed by the inhibitor to final concentrations of 3×10^{-3} or $1{\cdot}7 \times 10^{-2}$ M at arrows marked *p*-TS.

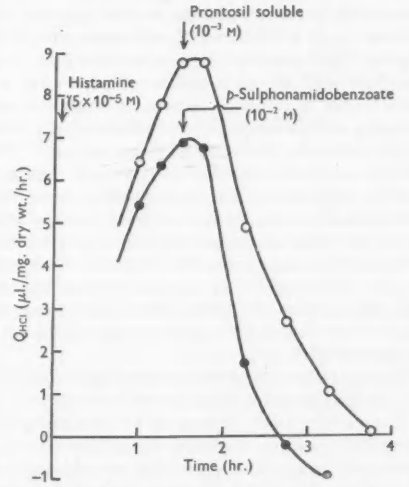

Fig. 2. Effect of 10^{-2} M-prontosil soluble and 10^{-2} M-*p*-sulphonamidobenzoate on histamine-stimulated acid secretion by isolated toad gastric mucosa.

The addition of *p*-toluenesulphonamide to final concentrations of 10^{-5}, 10^{-4}, 10^{-3} or 3×10^{-3} M had no observable effect on respiration or acid secretion by isolated gastric mucosa, but at 10^{-2} M the Q_{HCl} was lowered. In every case $1{\cdot}7 \times 10^{-2}$ M-*p*-toluenesulphonamide caused complete inhibition of acid secretion, but this always occurred after a lag period. There was usually only slight inhibition during the first 0·5 hr. after addition, but acid secretion was completely stopped within 1–1·5 hr. (Fig. 1). This delayed, but complete, inhibition of acid secretion was also observed with 10^{-2} M-*p*-sulphonamidobenzoate, 10^{-2} M-thiophene-2-sulphonamide and 10^{-3} M-prontosil soluble (Fig. 2), but not with 10^{-3} or 7×10^{-3} M-sulphanilamide. Not only was acid

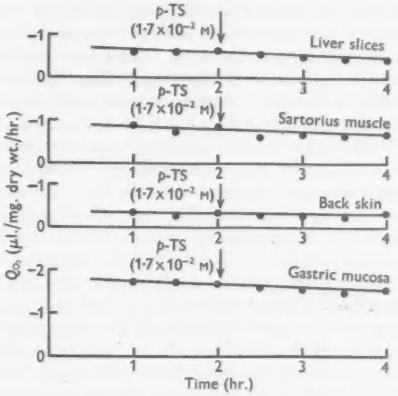

Fig. 3. Lack of effect of $1{\cdot}7 \times 10^{-2}$ M-*p*-toluenesulphonamide, added at arrows marked *p*-TS, on unstimulated respiration of some isolated toad tissues.

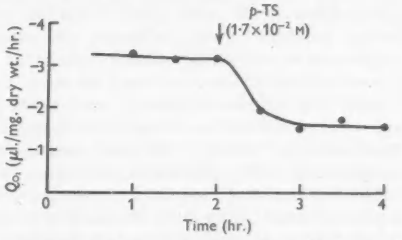

Fig. 4. Inhibition of respiration of toad-kidney slices following addition of $1{\cdot}7 \times 10^{-2}$ M-*p*-toluenesulphonamide at arrow marked *p*-TS.

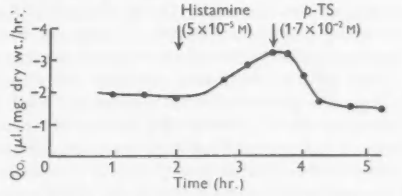

Fig. 5. Inhibition of histamine-stimulated respiration of isolated acid-secreting toad gastric mucosa following addition of $1{\cdot}7 \times 10^{-2}$ M-*p*-toluenesulphonamide at arrow marked *p*-TS.

secretion stopped, but the acid already secreted into the mucosal bag began to leak outwards (Figs. 1 and 2).

Effects on respiration and aerobic glycolysis. It was important to find out whether this inhibition of acid secretion was caused by some non-specific toxic action of these substances. The unstimulated respiration of isolated toad-liver slices, sartorious muscle, back skin, pancreas or gastric mucosa was found to be unaffected by 10^{-5}, 10^{-4}, 10^{-3}, 3×10^{-3}, 10^{-2} or $1{\cdot}7 \times 10^{-2}$ M-*p*-toluenesulphonamide (Fig. 3). In contrast with this the respiration of toad-kidney slices was found to be inhibited by $1{\cdot}7 \times 10^{-2}$ M-*p*-toluenesulphonamide (Fig. 4). The histamine-stimulated respiration of gastric mucosa was inhibited by $1{\cdot}7 \times 10^{-2}$ M-*p*-toluenesulphonamide in the way expected if the respiration of the oxyntic cells were abolished after a lag period in a similar fashion to the inhibition of acid secretion (Fig. 5). Experiments with open sheets of histamine-stimulated gastric mucosa in bicarbonate saline showed that $1{\cdot}7 \times 10^{-2}$ M-*p*-toluenesulphonamide increased the aerobic glycolysis. This increase in aerobic glycolysis also occurred in the presence of 10^{-2} M-thiophene-2-sulphonamide and 10^{-2} M-*p*-sulphonamidobenzoate, but was much smaller with prontosil soluble. Such increases in aerobic glycolysis are a common sign of cell damage.

If the incubation was continued for more than 1 hr. after the addition of these powerful carbonic anhydrase inhibitors to actively acid-secreting gastric mucosa, ulcerations and perforations occurred which were indistinguishable from those described by Davies & Longmuir (1948) for active mucosa stimulated to secrete hydrochloric acid in the absence of external supplies of carbon dioxide.

Effects on carbonic anhydrase activity. The carbonic anhydrase content of the gastric mucosa of frogs and toads used was found to be from 0·1–0·2 Davenport unit/mg. wet wt. This is similar to the values found for frogs by Davenport (1946*b*). Numerous estimations were made of the carbonic anhydrase activity of dilute and concentrated extracts prepared by grinding the gastric mucosa with sand after the incubations with inhibitor. In no case where acid secretion had been abolished was any trace of activity found. This failure to detect any activity, by the method of Krebs & Roughton (1948), in concentrated extracts of gastric mucosa means that the carbonic anhydrase must have been inhibited more than 99 %.

Although prontosil soluble is highly coloured and is an exceedingly powerful inhibitor of carbonic anhydrase, it was not found possible to use it to determine the distribution of this enzyme in the oxyntic cells, because this compound stains proteins unspecifically and was diffusely distributed in the mucosa.

DISCUSSION

Although sulphonamides inhibit carbonic anhydrase solutions instantaneously (Mann & Keilin, 1940), it is of interest that the effects on acid secretion became apparent only after a lag period. Carbonic anhydrase is needed to catalyse the hydration of carbon dioxide and hence prevent the accumulation of alkali in acid-secreting oxyntic cells. The resultant bicarbonate ions are then exchanged for chloride ions from the medium. Complete inhibition of the enzyme should thus lead to the disorganization of acid-secreting oxyntic cells after a time lag, and this actually occurs.

Mann & Keilin (1940) showed that the type of sulphonamide used in these experiments does not inhibit respiration, glycolysis or a variety of enzyme systems, even in very high concentrations. They wrote: 'These inhibitors therefore make it possible to abolish the activity of carbonic anhydrase in cells and organisms without affecting the activity of other known catalytic systems.' The unstimulated respiration of frog-liver slices, sartorius muscle, back skin, pancreas or gastric mucosa was unaffected by the inhibitors, and in these cases carbonic anhydrase was either absent or non-functional. The respiration of acid-secreting gastric mucosa was lowered by the amount expected if damage to the oxyntic cells followed the inhibition of carbonic anhydrase (Fig. 5). These results, together with the increase in glycolysis and the appearance of ulceration and perforations in mucosae which had been actively secreting acid, show that the inhibitors only affect gastric mucosa when it is secreting acid. This strongly supports the view that their action is entirely dependent on their ability to inhibit carbonic anhydrase. It is of interest that the ulcerations and perforations observed when actively acid-secreting mucosae were deprived of external supplies of carbon dioxide can also appear when the external supplies of carbon dioxide are made unavailable to the oxyntic cells by the inhibition of carbonic anhydrase.

The inhibitors are needed in very high concentrations judged by their effectiveness in free solution at 0° (Davenport, 1945; Krebs, 1948). This is expected for several reasons. Carbonic anhydrase activity is present in many cells in great excess and high concentrations of inhibitor have been needed to demonstrate the effects of the inhibition of carbonic anhydrase on carbon dioxide evolution from blood *in vitro* and *in vivo* (Keilin & Mann, 1941; van Goor, 1944; Jacobs & Stewart, 1942; Anderson & Thomson 1948) and on acid secretion and ammonia excretion by kidney (Höber, 1942; Pitts & Alexander, 1944; Pitts & Lotspeich, 1946; Ferguson, 1951). For blood *in vitro*, Davenport (1946*a*) has calculated that if less than 97 % of the enzyme is inhibited the cata-

lysed reaction is entirely uninhibited, and to reduce the catalysed rate by 90 % more than 99·8 % of the enzyme must be inhibited. The fact that it is more difficult to show effects of inhibiting carbonic anhydrase in the stomach than in the kidney and lungs may be partly because 'in the kidney as in the lungs the rate of flow of a fluid through a tube sets a time limit within which the required series of reactions must occur' (Davenport, 1946*a*). Another factor is the large excess of carbonic anhydrase activity even in oxyntic cells which are secreting acid at high rates (Davies & Roughton, 1948), and the fact that the uncatalysed hydration of carbon dioxide within oxyntic cells is sufficiently fast on its own to maintain the acid-base balance during rates of acid secretion of the order of one-tenth the maximum observed rates.

In the non-secreting oxyntic cells, as in other cells, carbon dioxide evolution occurs because the cytoplasmic pH is lower than the equilibrium value: in the acid-secreting oxyntic cell carbon dioxide uptake occurs because the cytoplasmic pH is higher than the equilibrium value, since hydrogen ions are continuously removed to form hydrochloric acid. The amount of carbonic anhydrase activity needed within acid-secreting oxyntic cells depends on the extent to which the pH of the cytoplasm is alkaline to the equilibrium value. Davies & Roughton (1948) have calculated that with a Q_{HCl} of 500 in mammalian cells at a pH difference of 0·01 a magnification of the uncatalysed uptake of 400-fold is required, whereas at a pH difference of 0·1 this is diminished to 40-fold. However alkaline the cell might become, a magnification of about ten is required. Since the carbonic anhydrase present is sufficient to produce a magnification of the order of 15,000-fold, a very high degree of inhibition (> 99·7 %) is needed to raise the cytoplasmic pH far enough to interfere with the enzymes involved in acid secretion.

Further reasons for needing high concentrations of these inhibitors are that the presence of protein lowers the effectiveness of these sulphonamides (Krebs, 1948), and that the carbonic anhydrase in the oxyntic cell may be less accessible to the inhibitors than carbonic anhydrase in free solution. Although no direct evidence is available regarding the percentage inhibition of the enzyme in the intact cells, there was no detectable enzyme activity liberated when the mucosae, in which acid secretion had been stopped, were ground with sand.

Sulphanilamide is from ten to one hundred times less effective than the other sulphonamides which were used (Krebs, 1948), and Feldberg *et al.* (1940) found that it did not inhibit acid secretion at 7×10^{-3} M concentration in decerebrate cats. This concentration is also ineffective in isolated frog gastric mucosa (Davies & Edelman, 1948). These experiments were carried out simultaneously with those of Davenport & Jensen (1948) who studied the effects of sulphanilamide and thiophene-2-sulphonamide on hydrochloric acid secretion by isolated mouse stomachs. They obtained a small but significant diminution of acid secretion with 0·0014 M-thiophene-2-sulphonamide and concluded that '...if carbonic anhydrase is necessary for acid secretion the quantity required is extremely small' (see also Davenport, 1946*a*). These observations are all in accordance with the theory advanced previously (Davies, 1948) that carbonic anhydrase plays a role in maintaining the acid-base balance in oxyntic cells during acid secretion.

Forster (1948), by observing the colour changes of phenol red, showed that isolated frog-kidney slices secrete acid even when unstimulated, and this finding has been confirmed in this laboratory. Davenport & Wilhelmi (1941) found large amounts of carbonic anhydrase in kidney, and it is known that inhibitors of this enzyme stop acid secretion in the frog kidney (Höber, 1942). The effect of *p*-toluenesulphonamide on respiration and acid secretion of frog and toad kidney slices is therefore expected (Davies, 1949).

SUMMARY

1. The presence of carbonic anhydrase in frog and toad gastric mucosa is confirmed.

2. Complete inhibition of hydrochloric acid secretion was produced, after a lag period, in isolated frog and toad gastric mucosa by the following concentrations of carbonic anhydrase inhibitors: $1·7 \times 10^{-2}$ M-*p*-toluenesulphonamide, 10^{-2} M-*p*-sulphonamidobenzoate, 10^{-2} M-thiophene-2-sulphonamide, and 10^{-3} M-prontosil soluble, but not by lower concentrations or by 7×10^{-3} M-sulphanilamide.

3. The unstimulated respiration of frog and toad liver slices, sartorius muscle, back skin, pancreas or gastric mucosa was unaltered by these concentrations of the inhibitors.

4. The respiration of acid-secreting frog and toad gastric mucosa was inhibited by these compounds; also, after a lag period, by the amount expected if the acid-secreting cells were damaged following the accumulation of alkali within them. A similar inhibition of respiration occurred in toad kidney slices.

5. No trace of carbonic anhydrase activity, even in concentrated extracts of frog and toad gastric mucosa, could be found after these experiments: the extracted enzyme was inhibited more than 99 %.

6. The results are in accordance with the view that carbonic anhydrase plays a role in maintaining the acid-base balance in oxyntic cells during hydro-

chloric acid secretion, but that the amount of carbonic anhydrase required is only a very small proportion of the total potential activity.

We wish to thank Prof. H. A. Krebs, F.R.S., for advice during the course of this work. One of us (J.E.) is indebted to the Agricultural Research Council for a grant.

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Isolation of Unequivocal Uroporphyrin III

A FURTHER STUDY OF TURACIN

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No authentic specimen of uroporphyrin III has until now been prepared either synthetically or from natural material.

The first uroporphyrin to be isolated was that from urine of the congenital porphyria patient Petry. Fischer (1915) originally considered it to be a heptacarboxylic porphyrin, but later (Fischer & Hilger, 1925) established that it was an octacarboxylic acid which could be partially decarboxylated, losing four acid groups and yielding coproporphyrin I, by heating with hydriodic acid in acetic acid or with 1% hydrochloric acid at 180–190° (Fischer, 1916; Fischer & Zerweck, 1924). This reaction established that it belonged to the isomeric series derivable from aetioporphyrin I, and Fischer concluded that it had the structure, porphin-1:3:5:7-tetra-acetic acid-2:4:6:8-tetrapropionic acid since comparison with a synthetic '*iso*uroporphyrin I' having four methyl and four methylmalonic acid β side chains and with a similar porphyrin having succinic acid instead of methylmalonic acid groups (Fischer & Zischler, 1937; Fischer & Hofmann, 1937) eliminated these structures as constitutional possibilities.

Uroporphyrin I, isolated from bones and urine of congenital porphyria patients and from pearl oyster shells, *Pteria vulgaris* (Fischer & Haarer, 1932), and the pigment from turacin, stated to be uroporphyrin I (Fischer & Hilger, 1924), was the only uroporphyrin known (although the melting points of the methyl esters of different preparations were widely variable from about 273 to 293°) when almost simultaneously, Waldenström, Fink & Hoerburger (1935; see also Waldenström, 1934, 1935, 1936) and Mertens (1936, 1937) claimed that the urine of sufferers from acute porphyria contained another uroporphyrin, the octamethyl ester of which melted at about 258°, and in the products of the decarboxylation of which

coproporphyrin III could be identified. It was thus regarded as uroporphyrin III belonging to the aetioporphyrin III series of position isomers.

About the same time, Rimington (1936) reported the study in South Africa of a living bovine case of congenital porphyria and the chromatographic separation upon alumina with dioxan as solvent of its urine and bone uroporphyrins into a fraction with m.p. 293° and another melting at 260–261°. It was suggested that the latter might be uroporphyrin III.

The homogeneity of the Waldenström ester from acute porphyria urines was disputed by Grinstein, Schwartz & Watson (1945) and Watson, Schwartz & Hawkinson (1945) who claimed that in spite of an apparently constant melting point of the crystalline material, it could in some cases (Type A Waldenström ester) be resolved by chromatography upon calcium carbonate into uroporphyrin I (ester m.p. 284°), yielding coproporphyrin I on decarboxylation, and a heptacarboxylic porphyrin (ester m.p. 208°) which apparently belonged to the III series, since on decarboxylation it yielded coproporphyrin III. Even in the case of esters which were not resolvable by chromatography (Type B Waldenström esters), it was claimed that decarboxylation indicated the same mixture or molecular association of I and III series porphyrins. Support for these claims has been proffered by Prunty (1946) and McSwiney, Nicholas & Prunty (1950). It followed that the porphyrin described by Waldenström and Mertens was not to be regarded as uroporphyrin III. Fischer & Hofmann (1937) applied adsorption chromatography, after Waldenström's publication, to a large quantity of 'uroporphyrin I' from Petry urine and claimed to have separated it into a uroporphyrin I ester melting at 302° (corr. 311°) and an ester of m.p. 261° which they regarded as identical with Waldenström's uroporphyrin III (mixed melting point determination). It was not further characterized.

The uroporphyrin copper complex 'turacin' (Church, 1869), which forms the red pigment in the flight feathers of several *Turacos* and related species of birds, was studied by Fischer & Hilger (1923, 1924), who concluded that it was a derivative of uroporphyrin I (comparison of melting point with that of uroporphyrin I; decarboxylation not performed). A re-investigation of turacin by one of the present writers (Rimington, 1939) produced unequivocal evidence that it yielded coproporphyrin III on decarboxylation and had therefore to be regarded as a series III isomer.

The ester of the uroporphyrin obtained by removal of copper from turacin was very difficult to crystallize, as Fischer & Hilger had found previously, but it is clear that if it could be properly purified it would provide for the first time a reference sample of authentic uroporphyrin III.

The present investigation was undertaken with two objects in view: (1) to obtain pure uroporphyrin III from turacin and to characterize it chemically and physically, and (2) to search for other porphyrins accompanying uroporphyrin III in turacin preparations.

Below, we present evidence that the main porphyrin derivable from turacin (mixed from several species of bird, but see Rimington, 1939) is indeed uroporphyrin III, melting point of octamethyl ester 264° and yielding coproporphyrin III on decarboxylation; it is accompanied by another porphyrin analysing as an 8 carboxyl porphyrin, but with octamethyl ester of melting point 210°, which we suggest may be a porphin octa-acetic acid. When decarboxylation of this pigment was attempted, employing the usual conditions, it proved to be unstable, the reaction mixture being colourless (except for some suspended material) and exhibiting only a trace of fluorescence.

The preparation contained, in addition, small quantities of a dichroic porphyrin, resembling spectroscopically a phylloporphyrin, and a chlorin-like material. These are regarded as chemical artifacts arising during the sodium-amalgam reduction and re-oxidation employed to remove the copper from turacin. Similar products have been obtained by subjecting a synthetically prepared copper uroporphyrin I complex to the same procedures.

MATERIALS AND METHODS

Feathers. These were obtained from the same material as used by Rimington (1939). Since all eleven species of birds had been shown to contain turacin, yielding coproporphyrin III on decarboxylation, the feathers from the different specimens were mixed.

Paper chromatography was performed according to Nicholas & Rimington (1949, 1951).

Adsorbents and solvents were prepared according to Nicholas (1951). Unless otherwise stated, the MgO used was grade III, and the Al_2O_3, grade IV.

Melting points were recorded upon an electrically heated micro-melting point stage using a calibrated thermometer.

Spectral absorption was measured with a Beckman photoelectric spectrophotometer, model DU, or on a Beck-Hartridge reversion spectroscope.

Decarboxylations were carried out in sealed Pyrex tubes in a thermostatically controlled, covered oil bath.

EXPERIMENTAL

Preparation of turacin. The red portions of the feathers were defatted by extraction with ether in a Soxhlet apparatus. Weight 6·68 g. They were steeped in successive changes of 1% (w/v) aqueous NH_3 until the extract was practically colourless. The deep-red turacin solution showed two bands only; there were no bands in the region of 635 mμ.

(see later). By addition of dilute HCl to pH 3·1, the pigment was precipitated. It was collected on the centrifuge and reprecipitated twice more under the same conditions. The last supernatant fluid was of pale straw colour.

Removal of copper. The turacin was dissolved in 0·1 N-NaOH and 200 g. of 2·5% sodium amalgam added in small portions at a time, shaking on a machine between each addition. The colourless mixture was filtered through a folded paper and the porphyrinogen re-oxidized by drawing a stream of air through the liquid overnight. By adjustment to pH 3·1, the uroporphyrin was precipitated; it was purified by reprecipitating twice. The first and second supernatants were greenish brown in colour and showed a strong, broad absorption band at about 620 mμ. The third supernatant contained a trace only of this component.

Isolation of a chlorin-like pigment

The supernatant fluids, referred to above, from precipitation of the uroporphyrin were combined and shaken with kieselguhr which adsorbed the substance having the strong absorption band in the red. The kieselguhr was washed, then

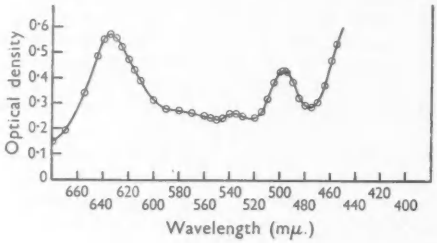

Fig. 1*a*.

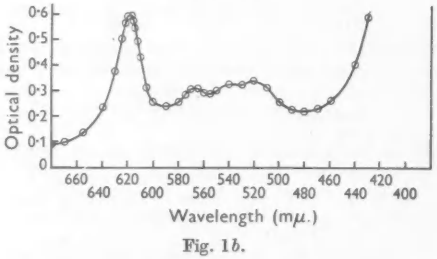

Fig. 1*b*.

Fig. 1. Spectral absorption of chlorin-like pigment isolated from mother liquors after isoelectric precipitation of turacin porphyrin. (*a*) In 0·1 N-NaOH; (*b*) in dilute HCl.

the pigment eluted with dilute ammonia, affording a greenish solution. Shaking with ethyl acetate removed the pigment from the acidified solution (pH 3·1) and from this solvent it passed back into dilute HCl. It was readsorbed upon a small quantity of kieselguhr (pH 3·1) and, after drying, this was treated with methanol saturated with HCl to esterify the pigment. The ester was extracted with $CHCl_3$ in the usual way and crystallized from $CHCl_3$-methanol. Recrystallization from benzene-light petroleum afforded greenish-brown needles. The spectral absorption of the free pigment was measured in 0·1 N-NaOH and in dilute HCl (see Fig. 1). The high band in the red in both acid (619 mμ.) and alkaline (635 mμ.) media suggests a chlorin structure for this material. Paper chromatography revealed the presence of material with 8 carboxyl groups. In addition to the two fluorescent spots characteristic of uroporphyrin (Nicholas & Rimington, 1949) this pigment and many of the others encountered in the present study of turacin exhibited a third faint spot lying below but very close to the uroporphyrin pair, i.e. there was a triple rather than a double spot system. The third spot was not sufficiently far down to correspond to a 7 carboxyl porphyrin.

Formation of a chlorin-like pigment by reduction of synthetic copper uroporphyrin

As it seemed likely that the chlorin could have been formed during the reduction of natural turacin, a similar experiment was carried out upon chemically prepared copper uroporphyrin. 1·50 g. of 2·5% sodium amalgam was added to the pigment dissolved in 25 ml. of 0·1 N-NaOH. After filtration, air was drawn through the solution until reoxidation had occurred and the porphyrin was precipitated at pH 3·1. The supernatant fluid was greenish and contained the same chlorin-like material as previously encountered (red band at 635 mμ. in 0·1 N-NaOH) together with some unprecipitated uroporphyrin.

Esterification and preliminary purification of the crude porphyrin

The precipitated porphyrin was dried *in vacuo* and left for 12 hr. in contact with methanol saturated with HCl. An equal volume of $CHCl_3$ was then added, together with sufficient saturated aqueous sodium acetate (rapidly) to neutralize the mixture to congo red. Much water was then added, and after thorough shaking the $CHCl_3$ layer removed, washed with dilute ammonia, then water, filtered and evaporated to dryness. The residue of crude ester (475·3 mg., m.p. 253°) was chromatographed upon a column of MgO packed in $CHCl_3$, using $CHCl_3$-methanol (100:0·5) for development. A small, dark residue was left on the top of the column and discarded, the porphyrin passing through with an indication of separation into two zones. It was recovered from the solvent, and rechromatographed upon a large MgO column (1 ml. adsorbent/mg. porphyrin) again using 100:0·5 $CHCl_3$-methanol. Three zones appeared: I, a lower band containing the majority of the pigment; II, an intermediate, rather diffuse band; and, III, an upper zone surmounted by a small layer of non-porphyrin material on the surface of the adsorbent. Each of these fractions was further purified, as described below (see also Fig. 2).

Purification of zone I. Isolation of turacin porphyrin A

This material was rechromatographed three more times on MgO. The porphyrin moved as a compact, mobile band leaving in the first two columns a slightly greenish residue on the adsorbent. The porphyrin was then crystallized from hot $CHCl_3$-methanol, affording rather poorly formed hair-like needles, m.p. 257–262°. Successive recrystallizations from benzene-light petroleum afforded a product with constant m.p. 264° and good crystalline form (see Fig. 3), as follows: 1st recrystallization from benzene-light petroleum, m.p. 259–264°; 2nd recrystallization from benzene-light petroleum, m.p. 264°; 3rd recrystallization from benzene-

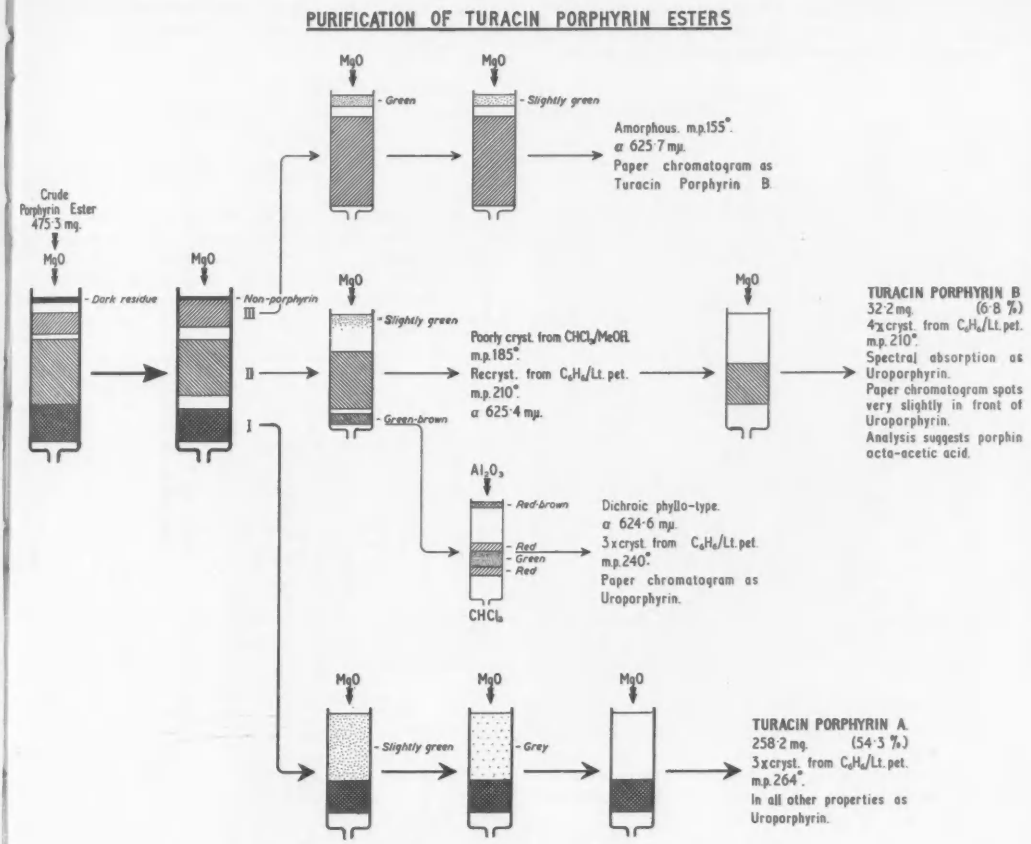

Fig. 2. Schematic representation of purification of turacin porphyrin.

Table 1. *Absorption maxima and relative intensities of turacin porphyrin esters A and B compared with those of uroporphyrin I ester and a Waldenström ester*

	Band I		Band II		Band III		Band IV		Soret band	
	mμ.	Relative intensity	mμ.	Relative intensity	mμ.	Relative intensity	mμ.	Relative intensity	mμ.	Relative intensity
Turacin porphyrin A ester, m.p. 264° (uroporphyrin ester III)	625	0·275	570	0·431	535	0·587	502	1·000	406	13·72
Uroporphyrin ester I, m.p. 293°, from bone	625	0·258	570	0·445	535	0·606	500	1·000	406	13·74
Waldenström ester, m.p. 260°, from acute porphyria urine	625	0·252	570	0·445	535	0·594	501	1·000	406	13·55
Turacin porphyrin B ester, m.p. 210°	625	0·268	570	0·491	535	0·625	502	1·000	406	13·72

light petroleum, m.p. 264°. This, the main constituent of the crude turacin porphyrin ester, weighed 258·2 mg. (54·3 % of the total crude porphyrin ester), and after hydrolysis behaved on paper chromatography as a single 8 carboxyl porphyrin indistinguishable from uroporphyrin I (see Fig. 4).

Fig. 3. Crystals from benzene-light petroleum of ester of turacin porphyrin A (uroporphyrin III octamethyl ester) m.p. 264°. Magnification ×250.

In elementary and methoxyl analysis it agreed with uroporphyrin octamethyl ester (Table 2). A spectrophotometric absorption curve of the ester in $CHCl_3$ (Fig. 5) showed no significant difference from either that (see Fig. 6) of uroporphyrin I octamethyl ester (m.p. 293°) from bones of a bovine case of congenital porphyria (Rimington, 1936) or that of a Waldenström ester (m.p. 260°) from acute porphyria urine (Rimington & Sveinsson, 1950). The ratios of the band intensities were as shown in Table 1.

Purification of zone II (see Fig. 2). Isolation of turacin porphyrin B and a dichroic pigment

Zone II, the middle zone in the second chromatogram of the crude turacin porphyrin (see Fig. 2), was rechromatographed upon MgO. It separated into a small, greenish-brown lower band which eventually yielded the dichroic pigment (see below) and a main, rather broad band. The upper part of the MgO retained a small quantity of greenish material, possibly similar to the chlorin already described, which was obtained from the precipitation mother liquors of the total crude porphyrin.

Turacin porphyrin B. The material from the main band crystallized poorly from hot $CHCl_3$-methanol. In $CHCl_3$ it had α-band at 625·4 mμ. It was rechromatographed once more upon MgO, on which it behaved as a single band,

Fig. 4. Filter paper chromatogram (upper half) of turacin porphyrin A compared with (lower half) uroporphyrin I, coproporphyrin I and deutero-porphyrin IX as markers.

and then recrystallized four times from benzene-light petroleum when the melting point became constant at 210°. This material weighed 32 mg. (6·8 % of the total crude porphyrin ester), thus corresponding to about one-tenth of the weight of turacin porphyrin A. After hydrolysis its R_F value by paper chromatography was consistently (nine determinations) slightly, but significantly, lower than that of the uroporphyrin marker and turacin porphyrin A. Micro-analysis (see Table 2) agreed better with the requirements of a porphin

Table 2. *Micro-analysis of turacin porphyrin esters A and B*

	C (%)	H (%)	N (%)	MeO (%)
Turacin porphyrin A ester (uroporphyrin III ester)	61·9	6·4	5·8	26·5
Required for uroporphyrin octamethyl ester $C_{48}H_{54}O_{16}N_4$	61·1	5·8	5·9	26·3
Turacin porphyrin B ester	59·5	6·1	6·1	28·1
Required for a porphin octa-acetic acid methyl ester $C_{44}H_{46}O_{16}N_4$	59·6	5·2	6·3	28·0

octa-acetic acid ester than with that of a uroporphyrin ester (porphin-tetra-acetic acid tetrapropionic acid ester). The behaviour of this material on decarboxylation (see below) affords some support for this suggested structure. The spectral absorption of the methyl ester (see Fig. 7) was not significantly different from that of uroporphyrin ester

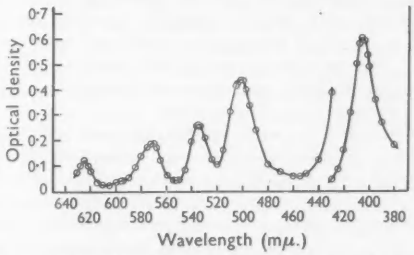

Fig. 5. Spectral absorption of ester of turacin porphyrin A (uroporphyrin III octamethyl ester). Solvent, chloroform. Concentration 28·13 mg./l. for range 630–430 mμ., 2·81 mg./l. for Soret band.

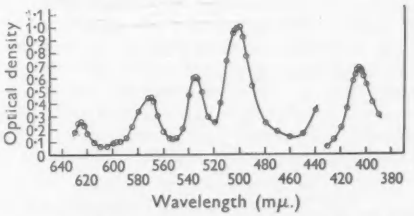

Fig. 6. Spectral absorption of uroporphyrin I octamethyl ester, m.p. 293° (see Rimington & Sveinsson, 1950). Solvent, chloroform. Concentration 65·8 mg./l. for range 630–440 mμ., 3·29 mg./l. for Soret band.

(Beckman spectrophotometer). The band ratios are recorded in Table 1. Comparison of the infrared absorption spectrum with those of uroporphyrin esters I and III (Falk & Willis, 1951), the latter provided by this work, supports the suggestion that turacin porphyrin B is closely related to, but not identical with, either of these uroporphyrins.

Dichroic pigment

The greenish-brown band obtained in the purification of zone II was rechromatographed upon Al_2O_3 packed in benzene. Development only began with pure $CHCl_3$ when a green band with red upper and lower edges moved down, leaving a small red-brown residue on the surface of the adsorbent. This residue was discarded and the green band eluted with methanol. Transferred again to $CHCl_3$, it had an α-band at 624·6 mμ. and was distinctly dichroic (red-green). It crystallized from $CHCl_3$-methanol in feathery greenish-

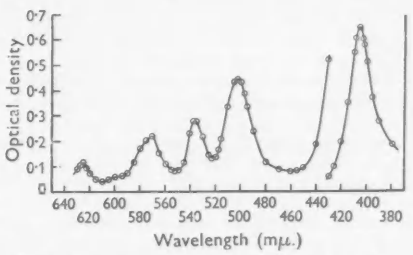

Fig. 7. Spectral absorption of octamethyl ester of turacin porphyrin B. Solvent, chloroform. Concentration approx. 30 mg./l. for range 630–430 mμ., 3 mg./l. for Soret band.

brown crystals, m.p. 212°. On recrystallization (three times) from benzene-light petroleum, the melting point became constant at 240°. The paper chromatogram of the ester after hydrolysis was similar to that of a uroporphyrin, but the second spot had a greater relative intensity. Spectral

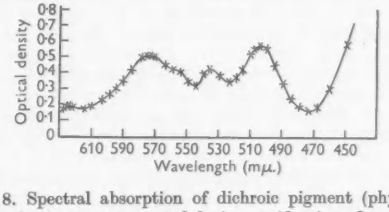

Fig. 8. Spectral absorption of dichroic pigment (phylloporphyrin type) separated during purification of turacin porphyrins. Solvent, chloroform.

absorption in $CHCl_3$, measured on the Beckman spectrophotometer, revealed a curve of phyllo-type (Fischer & Orth, 1937), band II being broad and intense; order of intensity IV, II, III, I (see Fig. 8). The α-band maximum lay at 625 mμ. We consider that this pigment is probably a phylloporphyrin with 8 carboxyl groups which may have arisen from the turacin porphyrin during the chemical manipulations employed to remove copper from the feather pigment.

Purification of zone III

This zone on the second MgO chromatogram of the total crude porphyrin was eluted by methanol containing 1% (v/v) of H_2SO_4. It was transferred to $CHCl_3$ and purified by two successive chromatograms upon MgO. The main porphyrin band was rather diffuse and left a small greenish zone upon the adsorbent. An unsuccessful attempt was made to secure crystalline material from benzene-light petroleum. From these solvents, the melting point rose continuously from 155°. A paper chromatogram showed similarity with turacin porphyrin B. In $CHCl_3$ it had α-band at 625·7 mμ. The amount was small.

Decarboxylation of turacin porphyrins

The acetic acid residue as a substituent on the pyrrole ring is unstable. The same is true to a less marked extent in the macrocyclic porphyrin system. Exploiting this property, Fischer & Zerweck (1924) showed that the uroporphyrin from Petry urine, when heated with 1% HCl in a sealed tube at 180–190° for 3 hr., lost 4 mol. of CO_2 and yielded coproporphyrin I. Since all four coproporphyrins have been synthesized, this reaction affords an excellent means of establishing to which aetioporphyrin series the parent substance belongs; uroporphyrin III would yield coproporphyrin III.

Turacin porphyrin A. The uroporphyrin ester from turacin porphyrin A (m.p. 264°) (3·8 mg.) described above, was decarboxylated by heating with 10 ml. of 1% HCl under the prescribed conditions. It was found most convenient to weigh out the ester, leave it to hydrolyse in 0·4 ml. of 25% HCl for 24 hr. and then to transfer the solution to the combustion tube with 9·6 ml. of water.

The reaction mixture contained some dark material which was filtered off and treated with glacial acetic acid to remove any adsorbed porphyrin, this extract being added to the main bulk from which the porphyrin was extracted with ether in the usual way, then from the latter into HCl. After precipitation and esterification, the total porphyrin in $CHCl_3$ was passed through a column of MgO and the purified coproporphyrin crystallized from a very small volume of $CHCl_3$ by addition of hot methanol. The crystals which separated had the habit of coproporphyrin III and m.p. 153°, remelt 179°. Recrystallization did not alter these melting points. By concentration of the first mother liquor, a further crop of similar crystals was obtained also melting at 153°, remelt 179°. Paper chromatography after hydrolysis showed only a 4 carboxyl porphyrin. The solution thus contained only coproporphyrin III, indicating that the parent uroporphyrin was the series III isomer alone. The yield of coproporphyrin III isolated was 68% of theory.

Turacin porphyrin B. On account of the small quantity of pigment available, only 2·5 mg. was taken for decarboxylation under the conditions described above. Examination at the end of the experiment showed that the substance had been degraded beyond the porphyrin stage, there being only a trace of fluorescence in the colourless solution.

DISCUSSION

The work reported here shows that by the removal of copper from turacin a mixture of porphyrins and porphyrin-like materials is obtained. These, by the aid of chromatography, have been separated. The ester of the main constituent, turacin porphyrin A, has m.p. 264°, remaining constant upon recrystallization. In elementary analysis, methoxyl content, spectral absorption and (after hydrolysis) behaviour on paper chromatography, it corresponds to the octamethyl ester of a uroporphyrin.

Decarboxylation afforded a single coproporphyrin, identified as coproporphyrin III by paper chromatography, spectral absorption and the melting point behaviour of its tetramethyl ester. That only coproporphyrin III was present was shown by the fact that there was no difference in melting point between the first crop of crystals separating and those obtained by further concentration of the mother liquors; moreover, their melting points were not changed by recrystallization of the respective fractions.

The main constituent of turacin is thus uroporphyrin III copper complex (confirming the earlier result of Rimington, 1939) and the ester, m.p. 264°, we have prepared provides for the first time an authentic specimen of uroporphyrin III octamethyl ester.

Accompanying this porphyrin and separable from it by chromatography is a second porphyrin, turacin porphyrin B, the ester of which we have obtained with constant m.p. 210°. Although this material resembles uroporphyrin octamethyl ester in such characteristics as paper chromatographic behaviour and spectral absorption, its elementary and methoxyl analysis agrees more nearly with the requirements of a substance containing four CH_2 groups less than uroporphyrin ester, such as, for example, the octamethyl ester of porphin octaacetic acid. Difference from uroporphyrin is shown by the infrared spectrum and by the greater instability of this porphyrin under the conditions usually employed for decarboxylation. Only a trace of material exhibiting red fluorescence in ultraviolet light was recoverable from the reaction mixture. An acetic acid grouping attached to a β-position in the pyrrole ring is known to be unstable. Fischer & Müller (1937) synthesized 1:4:5:8-tetramethylporphin-2:3:6:7-tetra-acetic acid which proved to be less stable than uroporphyrin. On decarboxylation under the usual conditions, octamethyl porphin was obtained, the yield of crude material being 20% of theory.

In the present instance, more complete degradation of the molecule appears to take place. Elucidation of the exact structure of turacin porphyrin B will be attempted by a synthetic approach. It is of interest to recall that from the urine of an atypical case of porphyria, Gray, Rimington & Thomson (1948) obtained an ether-insoluble porphyrin which suffered extensive degradation to colourless products when decarboxylation was attempted in acid solution. The presence of turacin porphyrin B,

together with uroporphyrin III in turacin from feathers, no doubt accounts for the extreme difficulty of crystallizing the methyl ester of the crude porphyrin obtained by the removal of copper from the natural pigment. In their first paper on turacin, Fischer & Hilger (1923) report that their porphyrin ester would not crystallize; in a later paper (Fischer & Hilger, 1924) they state that from 0·1 g. turacin they obtained a uroporphyrin ester in very small needles, but do not record the yield. The melting point was given as 295°, not depressed by admixture with uroporphyrin I octamethyl ester. This finding is difficult to understand.

During the purification of turacin porphyrins A and B, we also isolated a chlorin-like pigment and a dichroic pigment (ester m.p. 240°) with a phyllotype spectrum; both possessed 8 carboxyl groups by paper chromatography. We regard these as chemical artifacts arising during the reduction necessary to remove the copper from turacin. A similar chlorin-like pigment was obtained as a by-product by subjecting some uroporphyrin I copper complex to the same treatment with sodium amalgam as was accorded to turacin.

Now that an authentic specimen of uroporphyrin III octamethyl ester is available as a reference standard, it is hoped that some of the uncertainty surrounding the nature of the Waldenström porphyrin may be experimentally resolved. This pigment is excreted by sufferers from acute porphyria and was regarded as uroporphyrin III (see Waldenström, 1937) until Watson *et al.* (1945) disputed its homogeneity. Experiments with such an object in view have already been commenced.

SUMMARY

1. The porphyrin mixture resulting from the removal of copper from turacin has been examined chromatographically.

2. The main constituent has been isolated (ester m.p. 264°) and identified as uroporphyrin III. Decarboxylation afforded only coproporphyrin III. This provides for the first time an authentic specimen of uroporphyrin III.

3. Another porphyrin, present in about one-tenth of the amount, has been isolated as the methyl ester (m.p. 210°). From the results of analysis and paper chromatography it is suggested that this material may be porphin octa-acetic acid. When decarboxylation was attempted, it proved to be less stable than uroporphyrin, degradation into non-porphyrin products taking place.

4. In addition to these porphyrins, two materials having chlorin-type and phyllo-type spectra respectively have been isolated in very small quantity. They are, however, regarded as chemical artifacts.

The Beckmann photoelectric spectrophotometer used in this investigation was purchased out of a grant from the London University Research Grant Fund. One of us (R.E.H.N.) was in receipt of a grant from the Medical Research Council. Our thanks are due to Miss P. A. Miles for valuable technical assistance. The turacin was obtained from material given to one of us (C.R.) in 1938 by the British Museum (Natural History).

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A Study of the Porphyrins Excreted in the Urine by a Case of Congenital Porphyria

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Congenital porphyria is a rare disease. Most of our knowledge concerning it has been derived from investigations by Fischer and his collaborators upon the patient Petry and the study of the disease in cattle by Fourie, Rimington and their associates. Uroporphyrin I was first isolated from Petry's urine (Fischer, 1915*a*) and coproporphyrin I from his faeces (Fischer, 1915*b*). At the patient's death, a detailed chemical examination of his organs was carried out by Fischer, Hilmer, Lindner & Pützer (1925), while the pathological and fluorescence microscopical investigation was performed by Borst & Königsdörfer (1929). Examining some of the Petry urine uroporphyrin at a later date, Fischer & Hofmann (1937) obtained evidence of the presence of another porphyrin which they considered was uroporphyrin III. Rimington (1936) encountered a uroporphyrin, differing from uroporphyrin I, as a minor constituent of the South African bovine material.

Within recent years, the isotope-labelling technique has been applied both in England and in America (Gray & Neuberger, 1950; Gray, Neuberger & Sneath, 1950; Gray, Muir & Neuberger, 1950; London, Shemin, West & Rittenberg, 1949; Grinstein, Aldrich, Hawkinson & Watson, 1949) in an attempt to elucidate the relationship existing, in patients with congenital porphyria, between their urinary uroporphyrin and coproporphyrin, faecal urobilinogen and their blood haemin. No closer study has been made, however, of the types of porphyrin which they excrete.

The technique of paper chromatography applied to porphyrins (Nicholas & Rimington, 1949, 1951*a*) makes possible the recognition in very small amounts of material of pigments differing in the number of carboxyl groups they possess, and has revealed the presence in porphyrin mixtures from several natural sources of hitherto unknown porphyrins with seven, six, five or three carboxyl groups respectively. A careful study of porphyrin chromatography upon standardized adsorbents and using purified solvent mixtures (Nicholas, 1951) has greatly facilitated the separation and purification of individual constituents from crude mixtures.

The present paper describes the application of these techniques to a study of the porphyrins present in the urine of a case of congenital porphyria. Such urine provides a very convenient source of uroporphyrin I, the pure octamethyl ester of which may readily be obtained in quantity by following the procedures described.

EXPERIMENTAL

The patient was a male congenital porphyrinuric, first described by Mackey & Garrod (1926). He has also been the subject of some recent experiments with isotopically labelled glycine (Gray & Neuberger, 1950; Gray, Neuberger & Sneath, 1950; Gray, Muir & Neuberger, 1950). The urine examined (15·7 l.) was collected during August 1950, preserved with toluene, and sent to the laboratory. It contained 46·12 mg./l. of uroporphyrin, determined by the method of Rimington & Sveinsson (1950).

The urine was filtered, acidified with acetic acid (8 ml./l.) and left at 5° overnight. The precipitate was collected on the centrifuge. The supernatant retained a little porphyrin which was adsorbed upon kieselguhr then eluted by stirring with dilute ammonia. After precipitation and esterification followed by chromatography, there was recovered from this source 40 mg. of uroporphyrin octamethyl ester, m.p. 292°.

The main precipitate of porphyrin was suspended in 300 ml. of acetic acid and the mixture boiled under a reflux condenser for 5 hr.* When cold, it was filtered by suction through a layer of approx. 1 cm. of Celite 'Hyflo' kieselguhr supported by two Whatman no. 54 filter papers in a 7 cm. diameter Büchner funnel. The residue was washed, using minimal suction, by three lots of hot acetic acid, the last washing being colourless. These washings were added to the dark-brown original filtrate, the further examination of which is described below. The purified porphyrin was then eluted from the kieselguhr by drawing through it, under minimal suction, successive small quantities of N-NaOH. About 500 ml. was required to remove all the porphyrin. To the deep-red solution, sufficient concentrated HCl was added to bring the pH to 3·1, as determined by the glass electrode and after standing overnight at 5° the precipitated porphyrin was separated on the centrifuge from the pale straw-coloured supernatant and washed four times with N-acetic acid. It was then dried *in vacuo*. To the dry residue were

* We are greatly indebted to Dr J. Keilin for an outline of a method (Keilin, 1950) used by her for isolating uroporphyrin. From this method we have adopted the very convenient manoeuvre of boiling the crude porphyrin precipitate with acetic acid to remove coproporphyrin, but otherwise have followed our usual procedures which are here described in full.

added approx. 100 ml. of methanol, previously saturated with HCl gas, and the mixture left at room temperature for 24 hr. An equal volume of chloroform was then added and the mixture passed through a no. 3 sintered-glass filter. It was placed in a 1 l. separatory funnel and saturated aqueous sodium acetate solution added rapidly, mixing being achieved by swirling, until the colour change from purplish to ruby-red indicated neutralization of the mineral acid (pH > 3). About 500 ml. of distilled water was then added and the mixture was shaken thoroughly. The $CHCl_3$ layer was washed repeatedly with water, then by dilute ammonia, again with water, filtered and concentrated by distillation under reduced pressure.

To the hot concentrated solution were added approx. 3 vol. boiling methanol which caused immediate crystallization of the crude porphyrin ester. After chilling overnight, the crystals were collected on a no. 3 sintered-glass filter, washed with methanol, then ether and dried (wt. 712 mg.; m.p. 286°). The washings were added to the red mother liquor which was worked up separately (see below).

A paper chromatogram (Nicholas & Rimington, 1949, 1951*a*) on the crude uroporphyrin of m.p. 286° showed the presence of 8 carboxyl porphyrin (very strong), 7 carboxyl porphyrin (moderately strong) and approx. 6 carboxyl porphyrin (strong). Recrystallization from hot $CHCl_3$-methanol raised the melting point to 291°, but did not appreciably alter the paper chromatographic analysis.

Chromatographic purification of the crude uroporphyrins (see Fig. 1)

Isolation of uroporphyrin I. The material (712 mg.) was divided and chromatographed in two lots on MgO grade III (Nicholas, 1951) columns each of 17 × 5 cm., packed with $CHCl_3$. Development with 100 vol. $CHCl_3$/0·5 vol. methanol caused the greater part of the pigment to pass through the column as a compact band (*A* i). The pigment from this fraction was crystallized in typical hair-like crystals (634·4 mg., m.p. 293°) leaving an almost colourless mother liquor. The paper chromatogram revealed 8-carboxyl porphyrin only. (Micro-analysis: C, 61·4; H, 5·9; N, 5·4. Calc. for $C_{48}H_{54}O_{16}N_4$: C, 61·1; H, 5·8; N, 5·9%.) Decarboxylation afforded coproporphyrin I, m.p. 251–253°, unchanged by recrystallization (yield, 44% of theory). The material was thus pure uroporphyrin I ester. Continued development with a mixture of equal volumes of $CHCl_3$ and methanol caused the separation of two rather diffuse bands (*A* ii and *A* iii) which were collected together, leaving a deeply pigmented narrow layer (*A* iv) on the surface of the adsorbent. This could only be removed by methanol containing 1% (w/v) H_2SO_4. The pigment was transferred to $CHCl_3$ and crystallized by addition of boiling methanol, m.p. 289°, α-band 624·7 mμ. Rechromatography upon MgO afforded an ester crystallizing in the typical habit of uroporphyrin I (m.p. 292°). Experience has shown that

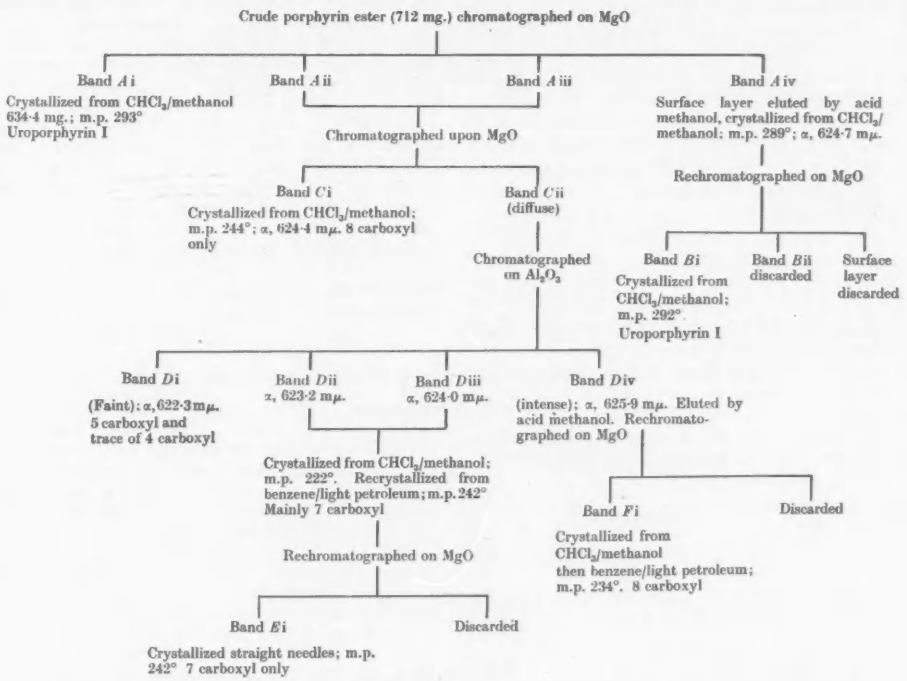
Crude porphyrin ester (712 mg.) chromatographed on MgO
Band A i
Crystallized from CHCl3/methanol 634·4 mg.; m.p. 293° Uroporphyrin I
Band A ii
Band A iii
Band A iv
Surface layer eluted by acid methanol, crystallized from CHCl3/methanol; m.p. 289°; α, 624·7 mμ.
Chromatographed upon MgO
Rechromatographed on MgO
Band C i
Crystallized from CHCl3/methanol; m.p. 244°; α, 624·4 mμ. 8 carboxyl only
Band C ii (diffuse)
Chromatographed on Al2O3
Band B i
Crystallized from CHCl3/methanol; m.p. 292° Uroporphyrin I
Band B ii discarded
Surface layer discarded
Band D i
(Faint); α, 622·3 mμ. 5 carboxyl and trace of 4 carboxyl
Band D ii
α, 623·2 mμ.
Band D iii
α, 624·0 mμ.
Band D iv
(intense); α, 625·9 mμ. Eluted by acid methanol. Rechromatographed on MgO
Crystallized from CHCl3/methanol; m.p. 222°. Recrystallized from benzene/light petroleum; m.p. 242° Mainly 7 carboxyl
Rechromatographed on MgO
Band F i
Crystallized from CHCl3/methanol then benzene/light petroleum; m.p. 234°. 8 carboxyl
Discarded
Band E i
Crystallized straight needles; m.p. 242° 7 carboxyl only
Discarded

Fig. 1. Chromatographic separation of esters of uroporphyrin I (m.p. 293°), an 8 carboxyl porphyrin (m.p. 234°), a 7 carboxyl porphyrin (m.p. 242°) and a 5 carboxyl porphyrin.

such zones of pigment which cannot be moved from the top of adsorbent columns are usually either unesterified material or are composed of porphyrin esters held there by impurities.

The diffuse bands (*A* ii and *A* iii) which were eluted together were rechromatographed on MgO. Development with 100 vol. $CHCl_3$/0·2 vol. methanol caused slow separation of a band from which an ester was crystallized in small hair-like needles (m.p. 244°, α-band 624·4 mμ.). Paper chromatography showed an 8-carboxyl spot only.

Isolation of porphyrins with 7 and 5 carboxyl groups respectively

Increasing the proportion of methanol to $CHCl_3$, after this band had been collected, removed the remainder of the pigment as a broad diffuse zone (*C* ii) passing slowly down the column. Paper chromatography showed this to be a mixture of porphyrins with 8, 7 and 6 or 5 carboxyl groups respectively. Further separation was effected by putting the material, dissolved in $CHCl_3$, on to a column of Al_2O_3, grade IV, packed in benzene and developing with $CHCl_3$. Four bands appeared, the lowest (*D* i, α, 622·3 mμ.) was faint, and although there was insufficient pigment for crystallization, paper chromatography showed it to be a 5 carboxyl porphyrin accompanied by a trace of coproporphyrin. The next band (*D* ii, α, 623·2 mμ.) was fairly strong, but was not sharply separated from the one above it (*D* iii, α, 624 mμ.). They were therefore combined and the material crystallized, first from $CHCl_3$/methanol (m.p. 222°) then from benzene/light petroleum, being obtained in straight needles, m.p. 242°. It was still heterogeneous, paper chromatography showing a 7 carboxyl porphyrin together with some 6 carboxyl and possibly also some 5 carboxyl porphyrin. Rechromatography upon MgO grade III gave a sharply defined lower band (*E* i) containing the 7 carboxyl porphyrin alone, as shown by the paper chromatogram; long straight needles, m.p. 242°.

The uppermost band (*D* iv) in the Al_2O_3 column from which these porphyrins were obtained was compact and intense (α, 625·9 mμ.). It was eluted by acid methanol, the pigment transferred to $CHCl_3$ and chromatographed upon MgO, grade III, which removed some brown material and afforded a porphyrin ester (*F* i) crystallizing in aggregates of hair-like needles and giving an 8 carboxyl spot on paper chromatography. The melting point was raised by recrystallization from benzene/light petroleum to 234°. This material

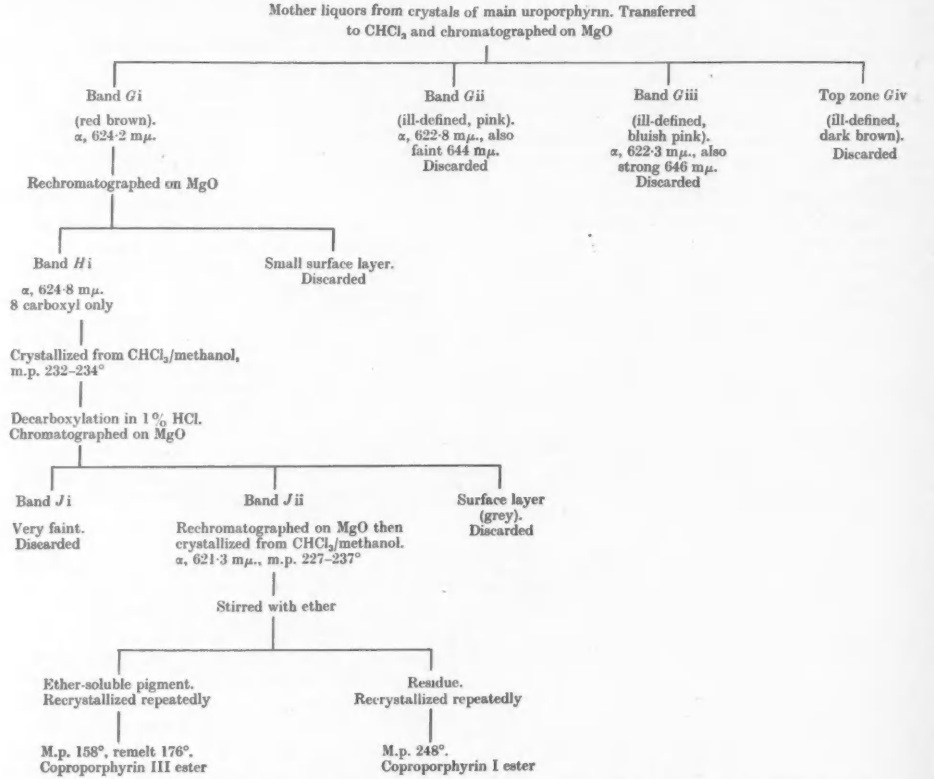

Fig. 2. Investigation of mother liquors of crystallization of main uroporphyrin I ester. Isolation of an association (m.p. 232–4°) of uroporphyrins I and III and its decarboxylation to coproporphyrins I and III.

was probably similar to that described in the preceding section with m.p. 244° and the material (m.p. 234°), the isolation of which is now to be described from the mother liquor of the first crystallization of the main uroporphyrin fraction.

Investigation of mother liquor from crystallization of main uroporphyrin fraction (see Fig. 2)

Isolation of an association of uroporphyrins I and III. The mother liquor from which the main crop of uroporphyrin (712 mg., m.p. 286°, see above) had separated, was deep red in colour. The pigment was transferred to $CHCl_3$ and placed on a column of MgO, grade III (15 × 2·5 cm.), which was developed with 100 vol. $CHCl_3$/0·6 vol. methanol. A complex chromatogram resulted with a rapidly moving brownish-red band (*G*i) surmounted by three less well defined zones (*G*ii, *G*iii and *G*iv) varying in colour from pink to bluish red. The α-bands of these fractions varied progressively from 622·8 to 622·3 mμ., but all exhibited absorption also at 644–646 mμ. *G*i was the only band investigated in detail. Rechromatography on MgO gave a compact band (*H*i) which separated from a small surface layer. It was collected (α, 624·8 mμ.) and found to contain only 8 carboxyl porphyrin by paper chromatography. Crystallization from $CHCl_3$/methanol afforded small aggregates of hair-like needles, m.p. 232–234°. As the quantity was small, it was all used for decarboxylation in 1% HCl at 180–190°. The resulting ether-soluble porphyrin was esterified and crystallized in aggregates of rather broad needles. For purification, it was chromatographed upon MgO, grade III, which removed some grey material and a minute, rapidly descending band (*J*i). The main band (*J*ii) was chromatographed again and the $CHCl_3$ solution (α, 621·3 mμ.) mixed with hot methanol. The crystals (m.p. 227–237°) left a deeply coloured mother liquor. Repeated recrystallizations from $CHCl_3$/methanol yielded typical coproporphyrin I ester (m.p. 248°). From the combined first and second mother liquors, coproporphyrin III ester (m.p. 158°, remelt 176°) was eventually obtained by recrystallizing the ether-soluble fraction of the dry residue from $CHCl_3$/methanol and then from benzene/light petroleum. The isolation of both coproporphyrins I and III indicates that the original 8 carboxyl material with m.p. 234° was an association of uroporphyrins I and III.

Investigation of the acetic acid extract of the crude porphyrin precipitate

Isolation of coproporphyrin I. The crude porphyrin precipitated from the urine by acidification had been boiled with acetic acid and then washed with hot acetic acid until this remained colourless. The combined extract and washings was brownish black in colour, and a test showed that all the porphyrin it contained was extractable by ether. Transference was effected into this solvent by addition of sodium acetate and much water. The ether layer was thoroughly washed and the porphyrin shaken out into 0·1 N-HCl. From this solution, it was precipitated at the isoelectric point, dried, esterified and chromatographed upon a column of MgO, grade III, packed in $CHCl_3$. Development with 100 vol. $CHCl_3$/1 vol. methanol eluted a main pink band (α, 621 mμ.) preceded by a narrow brown zone, and left a dark residue on the surface of the adsorbent. From the main eluate, the porphyrin was crystallized in curved needles (m.p. 246–248°; 4 carboxyl spot by paper chromatography). It was thus coproporphyrin I, possibly mixed with a small quantity of the series III isomer.

DISCUSSION

The application of standardized conditions of chromatography (Nicholas, 1951) and of paper chromatography (Nicholas & Rimington, 1949, 1951*a*) resulted in the detection and isolation from the urine of this congenital porphyrinuric of the following porphyrins: (1) uroporphyrin I, ester m.p. 293°; (2) a well crystallized 8 carboxyl porphyrin, ester m.p. 244°; (3) a crystalline 8 carboxyl porphyrin, ester m.p. 232–234°, possibly identical with the above but less pure. Decarboxylation of this material, which after hydrolysis gave only an 8 carboxyl spot by paper chromatography, afforded both coproporphyrin I (ester m.p. 248°) and coproporphyrin III (ester m.p. 158°, remelt 176°) proving that it was an association of the two uroporphyrin isomers I and III; (4) a 7 carboxyl porphyrin, the methyl ester of which crystallized in fine straight needles, m.p. 242°; (5) coproporphyrin I, ester m.p. 246–248° (possibly mixed with a little coproporphyrin III); (6) a 5 carboxyl porphyrin was also detected, but was not completely freed from accompanying coproporphyrin.

The yield of crude uroporphyrin I ester represented 91·7% of the total porphyrin present in the urine and 84% of the crude material was recovered as pure ester, after chromatographic removal of the 7 carboxyl and 5 carboxyl porphyrin esters (minimal in amount) and also the associations of 8 carboxyl porphyrin esters, presumably those of uroporphyrins I and III, which behaved chromatographically as entities and had melting points 234 and 244°. The quantities of these fractions did not exceed a few mg. The amount of coproporphyrin I ester ultimately isolated from the acetic acid washing of the crude porphyrin precipitate was 11·5 mg.

It is clear from the study of this case that the pattern of urinary porphyrin excretion in congenital porphyria may be more complex than has been previously realized. The association of uroporphyrins I and III in a complex, which crystallizes well and behaves as a chemical entity, is reminiscent of the claim by Grinstein, Schwartz & Watson (1945) and Watson, Schwartz & Hawkinson (1945) that the Waldenström esters (m.p. 255–260°) of acute porphyria urines are non-homogeneous. The above-named authors considered them to be associations of uroporphyrin I with a heptacarboxylic porphyrin (ester m.p. 208°) belonging to the III series. The evidence relative to the number of carboxyl groups in the latter is confined to an elementary analysis and methoxyl determination. The difficulty of obtaining small amounts of porphyrin esters in pure condition is great (cf. Fischer's

(1915*a*) original conclusion that uroporphyrin was a 7 carboxyl porphyrin). In this laboratory we incline to the view that uroporphyrins I and III may form an association, which is possibly molecular in character, and that this will separate chromatographically from the remaining isomer, whichever happens to be in excess. Authentic uroporphyrin III has been prepared from another source (Nicholas & Rimington, 1951*b*) and has an ester m.p. of 264°. Pure uroporphyrin I octamethyl ester has been found consistently to have m.p. 293°.

Although the possibility cannot be denied that the porphyrin ester of m.p. 255° separated chromatographically by Fischer & Hofmann (1937) from Petry's urinary uroporphyrin was in fact the ester of uroporphyrin III, it would seem more likely, in view of the findings recorded in this paper, that the material in question was an association of the two uroporphyrin isomers. No decarboxylation experiments were described by Fischer & Hofmann (1937) to support their view that it was uroporphyrin III. Similarly, it seems probable that the material with m.p. 255° described by Rimington (1936) as crystallizing out of the mother liquors of his preparation of uroporphyrin I octamethyl ester from bovine congenital porphyria urine and also the ester (m.p. 260–261°) separated chromatographically during the same study were associations of the kind now reported. Indeed coproporphyrin I was identified at this time among the products of decarboxylation of one of these bovine pigments.

SUMMARY

1. An investigation has been made, using chromatographic methods, of the porphyrins present in the urine of a case of congenital porphyria.

2. The pure uroporphyrin I octamethyl ester (m.p. 293°) isolated represented 77 % of the total porphyrin of the urine.

3. Fractions were also isolated, with melting points 234 and 244°, behaving as entities on the chromatogram and consisting of 8 carboxyl porphyrins only. Decarboxylation yielded coproporphyrins I and III, hence these fractions are regarded as associations (possibly molecular) of uroporphyrins I and III. The view is held that in mixtures of esters of the two isomers these associations will form and separate chromatographically from the remaining isomer, whichever happens to be in excess. Reports from previous investigations are examined in the light of this suggestion.

4. Small quantities were also isolated of coproporphyrin I (ester m.p. 246–248°) and of a 7 carboxyl porphyrin with ester m.p. 242°.

5. A 5 carboxyl porphyrin was detected, but was not completely separated from accompanying coproporphyrin.

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The Branched-Chain Fatty Acids of Butterfat

1. THE ISOLATION FROM BUTTERFAT OF BRANCHED-CHAIN FATTY ACIDS WITH SPECIAL REFERENCE TO THE C_{17} ACIDS

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Although branched-chain saturated fatty acids have been found in the lipids of certain bacteria as well as in wool wax, they have not hitherto been reported as existing in natural glycerides, with the solitary exception of *iso*valeric acid found by Chevreul in 1817 (cf. Hilditch, 1947) in the head and jaw oil of the dolphin.

Recently, Hansen & Shorland (1950) observed that the methyl ester concentrates of C_{18} unsaturated acids of butterfat when oxidized with finely ground potassium permanganate in acetone solution (Armstrong & Hilditch, 1925) yielded a residual neutral saturated product which was liquid at room temperature. Concentrates of other C_{18} unsaturated acids, such as those from pig fat, had been found to yield a solid neutral product corresponding to methyl stearate. Further purification of this liquid neutral product from butterfat was effected by recrystallization from acetone at $-30°$ followed by fractionation in an electrically heated column packed with glass helices (cf. Longenecker, 1937). A combustion analysis of one of the fractions suggested the presence of a C_{19} branched-chain acid contaminated with traces of oxygenated acids (cf. Hansen & Shorland, 1950).

Subsequent to this preliminary investigation it was found that the suspected branched-chain constituents could be separated by hydrogenation of the methyl esters of the unsaturated acids and subsequent crystallization from acetone at $-30°$, under which conditions the methyl esters of the branched-chain acids remained in solution.

The purpose of this paper is, first, to describe the general technique employed in the isolation from butterfat of two isomeric C_{17} branched-chain fatty acids; and, secondly, to record their properties.

EXPERIMENTAL

A concentrate (4·4 kg.) of C_{18} methyl esters prepared from butterfat was crystallized three times from 10 vol. of acetone at $-30°$. (The butterfat used in this investigation was prepared from butter churned on 16. v. 47 and supplied to this Laboratory through the courtesy of Dr F. H. McDowall, Dairy Research Institute, Palmerston North, by the Rangitaiki Plains Dairy Co. Ltd., Whakatane, New Zealand.) The resulting acetone-soluble 'liquids' were hydrogenated at 150° and at atmospheric pressure, using as catalyst nickel supported on kieselguhr. Repeated crystallizations from acetone at $-30°$ removed the 'solid' products of hydrogenation, and the acetone-soluble material (87·6 g. iodine value 8·3) was fractionated at about 0·01 mm. in an 8 ft. (2·4 m.) Vigreux column containing approximately twenty-two theoretical plates (cf. Table 1).

Fractions EL3, EL4 and EL5 were bulked together as methyl esters (33·0 g.), denoted Ea, and fractionated in the 8 ft. Vigreux column. Fractionation data are shown in Table 2.

Analysis of liquid methyl ester fraction EaL5 by Dr T. S. Ma, Microchemical Laboratory, Otago University, Dunedin, New Zealand. (Found: C, 75·8; H, 12·9. Calc. for $C_{18}H_{36}O_2$: C, 76·0; H, 12·8%.)

As a means of aggregating methyl esters of molecular weight similar to that of EaL5 (methyl esters of suspected C_{17} acids), selected fractions derived from EL1 to EL6 by repeated distillation were bulked together with EaL5, and the whole refractionated in the 8 ft. Vigreux column. On

Table 1. *Fractionation of* 87·6 *g.* C_{18} *liquid methyl esters*

Fraction	Wt. (g.)	Saponification equiv.	Iodine value	
EL1	2·25	269·7	7·3	Liquid
EL2	8·42	272·1	2·4	Solid
EL3	10·91	277·5	1·9	Semi-solid
EL4	10·38	283·1	3·2	Liquid
EL5	13·44	290·9	3·7	Liquid
EL6	14·05	308·2	4·9	Liquid
EL7	12·28	310·3	8·6	Liquid
EL8	2·45	317·6	23·0	Semi-solid
ELR*	14·03	263·4	25·7	—

* Residue (non-volatile). For esters in this fraction, excluding unsaponifiable matter, saponification equiv. 241·7; iodine value, 17·8.

Table 2. *Fractionation of Ea methyl esters*

Fraction	Wt. (g.)	Saponification equiv.	Iodine value	
EaL1	2·16	273·0	2·4	Solid
EaL2	4·83	275·5	1·1	Solid
EaL3	5·42	278·1	0·5	Solid
EaL4	3·61	280·3	0·6	Liquid
EaL5	8·19	284·7	1·4	Liquid
EaL6	6·35	291·4	3·1	Liquid
EaLR*	2·57	302·6	15·3	—

* Residue (non-volatile). For esters in this fraction, excluding unsaponifiable matter, saponification equiv. 299·9; iodine value, 12·8.

Table 3. *Chemical and physical characteristics of fraction Es*

	M.p.	Saponification equiv.	Iodine value	Refractive index
Es methyl ester	8·5–9·0°	284·9	1·5	$n_D^{22·5°}$ approx. 1·4419
Es acid	47·3°	—	—	$n_D^{60·2°}$ 1·4400

Table 4. *Fractional crystallization of Es acids from 40 vol. of methanol*

Fraction	Wt. (g.)	M.p. (°)	Temp. of crystallization (°)	Saponification equiv.
Es	1·59	47·3	—	270·9 (acid)
EsL	0·21	28·1	−37	—
EsS	1·37	48·6		
EsSL	0·26	37·0	−30	—
EsSS	1·12	48·7		
EsS2L	0·10	34·5	−42	—
EsS2S	1·01	51·0		
EsS3L	0·07	33·3	−45	—
EsS3S	0·93	50·5		
EsS4L	0·14	36·0	−39	—
EsS4S	0·78	50·9		
EsS5L	0·17	39·7	−35	—
EsS5S	0·60	53·3		
EsS6L	0·18	48·5	−35	—
EsS6S	0·41	53·9		
EsS7L	0·14	51·7	−35	269·5 (acid)
EsS7S	0·28	54·4		

completion of eight systematic distillations, a series of fractions was obtained whose saponification equivalents approximated 284. These fractions, namely, EfL1 (285·3), EfL2 (282·9), EfL3 (281·9), EiL1 (284·4), EiL2 (283·0), EiL3 (284·4) and EiL4 (284·5), when bulked together and denoted Ep, provided 5·47 g. of methyl esters. Ep was crystallized from 20 vol. methanol at −70°, and the portion insoluble in methanol at −70° was then recrystallized from 20 vol. of methanol at −60°, yielding 3·27 g. of 'solids' (Er2), saponification equivalent 283·4 (methyl ester), $n_D^{20°}$, 1·4443.

Chromatographic adsorption of Er2 on activated alumina removed traces of impurities and provided sixteen fractions; of these, the following four, which had been eluted with light petroleum (b.p. 50–60°) were bulked together as methyl esters to constitute Es: Er2b 0·87 g., $n_D^{22·5°}$ 1·4421; Er2c 0·54 g., $n_D^{22·5°}$ 1·4418; Er2d 0·25 g., $n_D^{22·6°}$ 1·4418; and Er2e 0·17 g., $n_D^{22·7°}$ 1·4420.

Properties of fraction Es are shown in Table 3.

Purification of Es fatty acids was effected by repeated low-temperature crystallization from 40 vol. of methanol of successive fractions insoluble in this solvent at the temperatures recorded in Table 4.

The chemical and physical characteristics of the residual fatty acid fraction, EsS7S, insoluble in methanol at −35°, are shown in Table 6.

Table 5. *Fractional crystallization of Et acids from 40 vol. of light petroleum (b.p. 50–60°)*

Fraction	Wt. (g.)	M.p. (°)	Temp. of crystallization (°)
Et	0·90	—	—
EtL	0·21	1	−45
EtS	0·67	42	
EtSL	0·01	—	−45
EtSS	0·65		
EtS2L	0·15	37·2	−30
EtS2S	0·50	40·5	
EtS3L	0·05	35·8	−30
EtS3S	0·45	39·2	
EtS4L	0·05	37·6	−30
EtS4S	0·39	40·2	

Table 6. *Chemical and physical characteristics of fatty acids EuS and EsS7S*

Fraction	Saponification equivalent	M.p. (°)	C-methyl* (%)	Refractive index	Combustion* analysis	Iodine value	X-ray diffraction analysis (A.) Long spacing	Side spacings†
EuS (acid)	270·1	39·8	8·06	$n_D^{60·8°}$ 1·4333	C 75·6, H 12·8	2·0	33·1	4·78, 4·45, 4·18, 4·03, 3·84, 3·70
EuS (methyl ester)	—	3·5	—	$n_D^{20·7°}$ 1·4422	—	—	—	—
EsS7S (acid)	269·5	54·4	6·68	$n_D^{72°}$ 1·4268	C 75·8, H 12·3	2·4‡	33·9	4·91, 4·66, 4·38, 4·18, 3·94, 3·68
EsS7S (methyl ester)	—	18·0‡	—	$n_D^{20°}$ 1·4438	—	—	—	—

* Analyses by Drs G. Weiler and F. B. Strauss, Oxford, England.
† Side spacings include the 7th, 8th and 9th order of the long spacings.
‡ Determinations made after 0·01 g. of EsS7S had been removed by crystallization from light petroleum and melting point had been raised to 54·5°.

Those fractions soluble in methanol at the temperatures given in Table 4, were bulked together to constitute Et (0·90 g.). Fractional crystallizations of Et and of successive 'solid' fractions derived from it were carried out using 40 vol. of light petroleum (b.p. 50–60°) at temperatures ranging from −45 to −30°, as shown in Table 5.

Residual fraction EtS4S (0·39 g.), subsequently denoted Eu, was crystallized from 40 vol. acetone at −40°, yielding an acetone-soluble fraction weighing 0·04 g., m.p. 35·2°. The fraction insoluble in acetone at −40° (EuS) weighed 0·35 g. Its chemical and physical properties are recorded in Table 6.

Using a Philips Geiger X-ray spectrometer, diffraction measurements were made on the two substances EsS7S and EuS. Samples were melted on a glass slide and quickly cooled.

DISCUSSION

The combustion analyses, together with the saponification equivalents of EuS and EsS7S (Table 6), agree closely with the theoretical values for $C_{17}H_{34}O_2$, viz. C, 75·5; H, 12·7%; saponification equivalent 270·4; while the C-methyl values afford evidence of the presence of two methyl groups in each acid (cf. Ginger, 1944). X-ray diffraction measurements show that the long spacings of EsS7S (33·9A.) and of EuS (33·1A.) cannot be considered to correspond within the experimental error (± 0·5A.) to a straight chain C_{17} saturated acid (cf. Table 7).

On the other hand, the long spacing of EsS7S is consistent with this fraction, being a methyl branched-chain C_{17} or C_{18} saturated acid, and the value for EuS corresponds to that of a C_{17} methyl branched-chain saturated acid. A study of the side spacings for long-chain *iso* acids published by Arosenius, Ställberg, Stenhagen & Tägtström-Eketorp (1949), reveals that the value of 4·16 appears in three odd-numbered homologues, namely, the C_{13}, C_{15} and C_{17} acids. A corresponding value of 4·18 was obtained in the X-ray analyses of both the samples EsS7S and EuS.

It has been observed by Cason (1948), that the only form of single methyl side-chain fatty acid which has a melting point less than 10° below that of the corresponding normal isomer is the *iso* acid (branching group on the carbon next to the end of the chain). Weitkamp (1945), in his investigations on the fatty acids of wool grease, presents evidence to show that *iso* acids with fourteen or more even-numbered carbons always melt one degree or less below the normal isomer. Accepting the value of 59·2–60·5° (Cason & Winans, 1950) for the melting point of normal heptadecanoic acid, it appears that

Table 7. *X-ray long spacings of straight-chain, methyl* iso, *and methyl* anteiso *saturated fatty acids*

Carbon content of acid	Long spacings (A.) Straight-chain saturated fatty acids*	Methyl *iso* saturated fatty acids†	Methyl *anteiso* saturated fatty acids‡
C_{13}	30·0	26·2	—
C_{14}	31·60§	27·0	—
C_{15}	34·2	29·8	—
C_{16}	35·60§	30·4	—
C_{17}	38·6	33·35	33·4
C_{18}	39·75§	33·8	—
C_{18} (*d*)	—	—	34·2
C_{18} (*d*–1)	—	—	28·0
C_{19}	43·15	36·2‖	—
C_{20}	44·15§	—	—

* Data by Francis, Piper & Malkin (1930).
† Data by Arosenius, Ställberg, Stenhagen & Tägtström-Eketorp (1949).
‡ Data by Velick (1947).
§ Data by Francis & Piper (1939).
‖ Data by Velick (1944).

EsS7S (m.p. 54·4) is an impure *iso* acid, 15-methylhexadecanoic acid. A further attempt at purification of EsS7S by fractional crystallization from light petroleum (b.p. 50–60°) at −40° removed in the soluble fraction only 0·01 g. and raised the melting point of the insoluble fraction 0·1°. Location of the position of the CH_3 side chain in fraction EuS is

under investigation, the melting point evidence (m.p. 39·8°), however, is consistent with the presence of a methyl side chain in the antepenultimate position (cf. Weitkamp, 1945) suggesting that EuS may be identified as 14-methylhexadecanoic acid (m.p. 36·8°).

Although data which have so far been obtained in this work are limited to the C_{17} acids, accompanying evidence indicates the presence in butterfat of a wide range of branched-chain fatty acids. Investigation of these acids is in progress.

Prior to the present investigation, no naturally occurring *iso* acid with an odd number of carbon atoms has been reported (cf. Arosenius *et al.* 1949), although two such odd-numbered *iso* acids, a C_{19} (Cason, 1942; Weitkamp, 1945), and a C_{17} (Weitkamp, 1945) have been synthesized.

The butterfat from which the two C_{17} branched-chain acids have been isolated was drawn from a mid-May (1947) churning of cream, representative of numerous cows in the Whakatane district. In our preliminary experiments, when the presence of branched-chain acids was suspected, the bulked C_{18} unsaturated methyl esters which yielded liquid saturated products on oxidation were derived from several churnings made during different months, suggesting that methyl side-chain acids are not peculiar to one butterfat sample only.

For the elucidation of the mode of combination of branched-chain acids in butterfat, further work is required. It has been shown by Morice (1951), that cholesterol comprises about 0·40 % of the total 0·65 % of unsaponifiable matter in butterfat. Of the cholesterol present, about 0·26 % is in the free form (Morice, 1951). Branched-chain acids probably comprise more than 0·8 % of the total fatty acid content of the butterfat, this quantity having been extracted from a concentrate of the C_{18} unsaturated acids alone. Accordingly, combination of branched-chain acids with cholesterol only is not reconcilable with the proportions of these substances present. It is probable, therefore, that the branched-chain acids are present in the neutral fat as glycerides, or are combined with some water-soluble compound such as the disaccharide trehalose, as is the case in certain bacillary lipids (Anderson, 1941).

SUMMARY

1. Branched long-chain fatty acids formerly unidentified in natural glycerides have been isolated from butterfat.

2. Two C_{17} methyl branched-chain fatty acids, isomeric with normal heptadecanoic acid are shown to be present in butterfat.

3. Based solely on the melting point evidence of branched-chain fatty acids as presented by Cason (1948), one of the two C_{17} fatty acids isolated (m.p. 54·4°) appears to be the *iso* acid, while the other (m.p. 39·8°) appears to be the *anteiso* acid.

We are indebted to Miss N. J. Cooke for technical assistance. The X-ray diffraction analyses, together with their interpretation, were made by Mr M. Fieldes of the Soil Bureau, Department of Scientific and Industrial Research, Wellington, New Zealand.

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The Virulence-Enhancing Factor of Mucins

4. THE ROLE OF PARTICULATE INSOLUBLE MATTER AND A VISCOUS MEDIUM IN VIRULENCE ENHANCEMENT, WITH A REVISED ASSAY OF THE THIRD FACTOR INVOLVED

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In Part 3 (Smith, 1951) of this series a virulence-enhancing fraction of hog gastric mucin was separated from the insoluble particulate residue. This residue had an adjuvant action on the enhancement of the virulence by the active factor comparable with that already demonstrated (Smith, 1950*b*) for the viscosity of the original mucin. However, using a biological assay involving a medium of constant viscosity for injection, no residue was obtained completely free from virulence-enhancing activity, even after autoclaving and washing with hot water. The suggestion was made, therefore, that a viscous medium, together with the particulate residue, could impede the host defence mechanisms by themselves, quite apart from their probable action in delaying the absorption by the host of the active factor. This suggestion is supported by the work of Olitzki & Koch (1945), who reported the adjuvant action of kaolin, fuller's earth, Norit A and talcum on the small virulence-enhancing activity of 0·3 % agar for *Shigella dysenteriae*, *Escherichia coli* and *Bacterium typhosum* (*Salmonella typhosa*), and of Olitzki, Shelubsky & Hestrin (1946), who noted a similar action of kaolin on the activity of a number of carbohydrates, although the viscosities of the carbohydrate preparations used were not noted or measured. The work described in this paper shows that the suggestion is in fact true.

Thus one can no longer refer to the virulence-enhancing factor of hog gastric mucin; the activity is due to a combination of three factors, all of which are slightly active alone, and none of which can be singled out as having a much greater share in the combined activity than the other two. Nevertheless, in contrast with the particulate insoluble matter and the viscous medium, the third factor is more specific. Knowledge of its chemical nature would be extremely interesting, especially for comparison with that of various bacterial products which have been shown to have virulence-enhancing activity (Olitzki, 1948).

It was essential for further chemical fractionation to obtain a reliable assessment of this third factor, samples of which are still called (somewhat inaccurately) virulence-enhancing samples. Although an assay involving a simple injection of the sample, together with *Bact. typhosum*, would be desirable, this was found to be impossible; too large a quantity is needed to inject adequate batches of mice with the relatively high concentrations required (approximately 1 %, w/v). A constant amount of particulate residue in a medium of constant viscosity is, therefore, combined with the third factor, which then need be used in concentrations of the order of only 0·025 % (w/v). The necessary alterations in the technique of the assay are described in this paper.

EXPERIMENTAL

General details for the biological testing of samples have been given (Smith, 1950*a*). Only the modified preparation of the suspensions for injection is described here.

The effect of insoluble particulate matter and of a viscous medium in enhancing the virulence of Bact. typhosum *for mice*

Insoluble particulate matter. Aqueous suspensions of charcoal, kaolin, fuller's earth, talc, a sample of the residue separated from the virulence-enhancing sample at pH 10·5 as previously recorded (Smith, 1950*b*), and a sample of the same residue which had been autoclaved in aqueous suspension and washed with hot water, were used. Serial dilutions of these suspensions were made and mixed with the requisite number of *Bact. typhosum*. The suspensions were thoroughly shaken before each dose was removed for injection. The results are given in Table 1.

After heating to 60° for 0·75 hr. the unautoclaved residue from hog gastric mucin was grossly contaminated with sporing Gram-positive organisms. This probably accounts for the death of some mice after injection of this residue alone. The other samples tested were sterile or contained only one or two organisms in 0·2 ml.

Viscous media. Gum tragacanth, gum acacia, agar, Cellofas (a methylcellulose), and a sample of semi-pure blood group substance obtained from hog gastric mucin by a combination of the methods of Morgan & King (1943) and Smith (1950*b*) were used.

Viscosity determinations were made on the aqueous solutions in an Ostwald viscometer at 37° as

Table 1. *The effect of aqueous suspensions of insoluble particulate matter in enhancing the virulence of* Bact. typhosum *for mice*

(General details of the method of testing are given by Smith (1950*a*). Routine experiments on a large number of mice have shown that 5×10^6 *Bact. typhosum* suspended in tryptic meat broth kill 2–8 of a batch of 10 mice. 5×10^5 or less kill no mice. Details of bacterial counts which were always 0·8–1·2 × 5000 organisms are not included in the table.)

Nature of particulate matter	No. of *Bact. typhosum*	Death rate (%) in batches of 10 mice injected with the following strengths (%, w/v)						Repeat experiments using batches of 20 mice: Strength (%, w/v) injected	Death rate (%): No. *Bact. typhosum*	Death rate (%): 5000 *Bact. typhosum*
		20	10	5	2·5	2	1			
Charcoal	5000	90	80	50	10	—	—	7·5	5	95
	Nil	20	Nil	Nil	—	—	—			
Kaolin	5000	90	70	20	Nil	—	—	7·5	Nil	60
	Nil	20	Nil	10	—	—	—			
Fuller's earth	5000	100	90	70	10	—	—	4	Nil	40
	Nil	90	90	10	—	—	—			
Talc	5000	30	40	Nil	Nil	—	—	20	Nil	25
	Nil	10	Nil	Nil	—	—	—			
Residue insoluble at pH 10·5 from hog gastric mucin	5000	—	—	100	70	80	10	1·5	15	50
	Nil	—	—	100	80	30	Nil			
Same residue autoclaved and washed with hot water	5000	—	—	100	70	70	10	3	5	95
	Nil	—	—	50	10	Nil	Nil			

Table 2. *The effect of a viscous medium in enhancing the virulence of* Bact. typhosum *for mice*

(General remarks as in Table 1.)

Nature of material producing the viscous aqueous solution	Solutions A, of low viscosity*: Approximate strength (%, w/v) of solution injected with 5000 *Bact. typhosum*	Solutions A, of low viscosity*: Death rate (%) in batches of 20 mice	Solutions B, of high viscosity†: Approximate strength (%, w/v) of solution injected	Solutions B, of high viscosity†: Death rate (%) in batches of 20 mice: No *Bact. typhosum*	Solutions B, of high viscosity†: Death rate (%) in batches of 20 mice: 5000 *Bact. typhosum*
Tragacanth	0·1	Nil	0·3	Nil	25, 35
Blood group substance from hog gastric mucin	1·75	Nil	6·0	Nil	15, 55
Acacia	10	Nil	30	Nil	10, 40
Agar	0·16	Nil	0·35‡	Nil	10, 10
Cellofas	0·4	5, nil	1·2	Nil	10, 25

* Viscosity 6–9 times that of water, compared in an Ostwald viscometer, B.S.S. 2, vol. 8 ml. at 37°.
† Viscosity 8–12 times that of solutions A, compared in an Ostwald viscometer B.S.S. 3, vol. 23 ml. at 37°.
‡ The viscosity of agar solutions near the gelling concentration is difficult to control for a given concentration and one batch of mice was injected with 0·35% (w/v) agar of viscosity higher than that stated.

described by Smith (1951), before mixing with the *Bact. typhosum* suspension. The viscosities of the more concentrated solutions were not compared directly with that of water, but with those of the dilute solutions, as the viscometer necessary for the determination of these very viscid solutions was too large to give a reasonably accurate figure for water. Results are given in Table 2.

Insoluble particulate matter combined with viscous media. Suspensions of the six insoluble materials noted above, in concentrations which were inactive alone in aqueous suspension, were made in aqueous solutions of the five viscous materials used, at concentrations which had already been proved to be inactive alone. The suspensions of the insoluble matter and solutions of the viscous materials were heated to 60° for 0·75 hr. in double strength before mixing. As before, viscosities were determined before adding the *Bact. typhosum* suspensions. Results are given in Table 3.

Specificity of the third factor involved in virulence enhancement

Using the revised method of assay described below, virulence-enhancing samples from hog gastric mucin show definite activity at 0·0125% (w/v). Undegraded thymus deoxypentose nucleic acid, yeast pentose nucleic acid, crystalline pepsin,

Table 3. *The effect of a combination of insoluble particulate matter and a viscous medium in enhancing the virulence of* Bact. typhosum *for mice*

(General remarks as in Table 1. The death rates of adequate control batches injected without *Bact. typhosum* are not included in the table: these death rates were nil in nearly all cases and never more than 10% except for the unautoclaved residue from hog gastric mucin which showed a consistent death rate of 10–20% irrespective of the viscous medium in which it was injected. The viscosity of all suspensions was 6–9 times that of water compared in an Ostwald viscometer (B.S.S. 2, vol. 8 ml.) at 37°.)

Nature and approximate strength (%, w/v) of the material producing the viscous suspension	Death rates (%) in batches of 20 mice injected with 5000 *Bact. typhosum* and the following strengths (w/v) of the stated particulate matter in the various viscous media						
	None	Charcoal (2·5%)	Kaolin (2·5%)	Fuller's earth (2·5%)	Talc (5%)	Autoclaved residue from hog gastric mucin (1·5%)	Residue from hog gastric mucin (0·75%)
None	—	10	Nil	10	Nil	10	Nil
Tragacanth, 0·12	Nil	55	45	50	55	45	50
Blood group substance from hog gastric mucin, 1·75	Nil	100	65	65	30	90	70
Acacia, 10	Nil	95	30	40	20, 15	60	45
Agar, 0·16	Nil	80	70	80	60*	55	55
Cellofas, 0·4	5, nil	95	10, 25	20, 15	55	45	45

* Viscosity 5·1 times that of water at 37°.

crystalline trypsin, bovine albumen, human globulin, dialysed and undialysed acid and alkaline hydrolysates of virulence-enhancing samples showed no activity at 0·05% (w/v). In addition, various polysaccharides and mucopolysaccharides have been shown to act only by virtue of their viscosities.

The third factor involved in virulence enhancement is, therefore, to some extent specific. However, it would be interesting to test several bacterial products under the conditions of the assay.

Revision of the biological assay

In the original assay of virulence-enhancing activity (Smith, 1950*a*) samples were compared with a 'standard' mucin preparation consisting of a well homogenized batch of crude hog gastric mucin (Wilson's Granular Mucin; Smith, 1950*a*). This preparation gave a viscous solution and contained insoluble particulate matter as well as the more specific factor. Hence, the slope of the regression line of the probit of the death rate with dose, used for each comparison of 'standard' with unknown mucin, was a function of the variation of all the three factors which are now known to have virulence-enhancing activity. In the assay described below the unknown sample is compared in the same test with a new 'standard' preparation freed from the two non-specific factors, each being injected in the presence of a constant amount of particulate residue and in a suspension of constant viscosity. As was expected from the above considerations, the slope of the regression line of both the new 'standard' preparation and unknown samples (1·5, 1·0–2·0) was different from, and smaller than, that for the original 'standard' preparation (5·8, 5·1–6·3). Hence more batches of mice than in the original assay are needed to yield the accuracy aimed at—namely the detection with probability, $P = 0{\cdot}95$, of approximately a twofold difference in virulence-enhancing activity.

New 'standard' virulence-enhancing preparation. A batch (100 g.) of crude virulence-enhancing mucin was freed from materials yielding a viscous solution and from particulate residue by a modification of the methods given by Smith (1950*b*, 1951). It is kept over P_2O_5 at 0° and contains 5% moisture (loss at 0·1 mm. Hg and 60° over P_2O_5). It is injected into batches of 20 mice at concentrations of 0·4, 0·2, 0·1 and 0·05% (w/v), which, under the conditions of the assay, cover the death rate range 20–80%.

Unknown virulence-enhancing samples. The material is injected into batches of 20 mice at four concentrations (calc. for the substance dried at 0·1 mm. Hg at 60° over P_2O_5), at twofold intervals, which have been shown by preliminary experiments to cover the death rate range 20–80% under the conditions of the assay.

Particulate matter. This is a large batch of charcoal (SS 14) which has been thoroughly mixed and passed through a fine sieve (160 mesh). The constant concentration in suspension in all injected fluids is 0·4% (w/v).

Constant viscosity. The viscosity is maintained at seven to nine times that of water (Ostwald viscometer B.S.S. 2, 37°) by means of tragacanth solutions (0·13–0·18% (w/v) according to the particular mucin solution and its strength) as previously described. The finely divided charcoal does not interfere with the viscosity determination.

Preparation of the material for injection. The new 'standard' third factor, the unknown virulence-enhancing samples and the charcoal are made up at four times the strength ultimately required and adjusted to pH 7·0–7·2. The tragacanth solution is prepared at double strength and adjusted to the same pH. Equal quantities of virulence-enhancing sample and charcoal suspension are mixed and shaken with glass beads overnight to allow adsorption to take place. These

mixtures and the tragacanth solutions are heated separately to 60° for 0·75 hr., cooled and mixed in equal quantities. After determining the viscosities at 37° on separate samples, the requisite quantity of *Bact. typhosum* is added and the mixtures injected into batches of 20 mice. Particular care is taken that the suspensions have been well mixed before doses are removed.

In addition to the usual controls previously described (Smith, 1950*a*), in 18 experiments the suspension of charcoal in the medium of constant viscosity was injected into mice together with 5000 *Bact. typhosum*. Only 14 of 360 mice died, and in no batch of 20 mice did more than two die. This shows that the effect of the two non-specific factors had been sufficiently reduced.

Comparison of the death rates produced by the four concentrations of 'standard' and unknown virulence-enhancing samples. This was done by probit analysis. The activity of the unknown sample in terms of the 'standard' is expressed in units/g., the strength of the 'standard' being 1 unit/g. These units are arbitrarily called 'Virulence-enhancing units' as distinct from 'Wilson units' (Smith, 1950*a*). Mr S. Peto writes a note on the statistical treatment of the data as an addendum to this paper.

Table 4 shows some typical results, together with weighted means of duplicate assays. The fiducial limits show that with probability, $P = 0{\cdot}95$, a difference of virulence-enhancing power of 3·0–3·5 times is detected by one assay, and of 2·0–2·5 times by a combination of duplicate assays.

DISCUSSION

The results described by Smith (1950*b*, 1951), together with those in the first part of this paper, make it clear that no single chemical substance is responsible for the virulence-enhancing action of mucin. The activity is due to a combination of one factor which is relatively specific, with two that are non-specific. Each factor has some activity when used alone, but a much higher dose is needed than when it is combined with the other two. The differences between the minimum concentrations showing an effect for each agent acting alone (charcoal 7·5 % (w/v), viscosity ten to twelve times that in the active combination, third factor 1 %, w/v) and those for the agents in combination (charcoal 0·4 % (w/v) viscosity unity, third factor 0·025 %, w/v) show that the effect is synergic and not additive.

It is true that no one agent has a much greater share in the combined activity than the other two. Nevertheless, consideration of the yields and activities of the three agents from the crude mucin indicates that the relative effects are in the descending order third factor, particulate residue, and viscous medium. The difference between the relative effects of the first two is less than that between the last two.

Table 4. *The results of duplicate assays on various virulence-enhancing fractions together with their weighted means*

Virulence-enhancing fraction	Assay	Most probable result in 'Virulence-enhancing units'/g.	Fiducial limits ($P = 0{\cdot}95$)	
			Minimum	Maximum
588	1	2·5	1·2	4·3
	2	2·8	1·4	4·9
	Weighted mean	2·7	1·7	4·1
589	1	3·7	2·0	6·5
	2	8·0	4·8	14·7
	Weighted mean	5·6	3·7	8·4
590	1	4·5	2·6	7·6
	2	4·2	2·3	7·1
	Weighted mean	4·3	3·0	6·4
553	1	2·7	1·3	4·9
	2	1·4	0·7	2·4
	Weighted mean	1·9	1·2	3·0
554	1	3·9	2·2	7·2
	2	3·5	2·0	6·1
	Weighted mean	3·7	2·4	5·5
641	1	2·3	1·5	3·6
	2	3·7	2·3	5·8
	Weighted mean	2·9	2·1	3·9
642	1	3·4	2·2	5·4
	2	3·6	2·2	5·9
	Weighted mean	3·5	2·5	4·8

Knowledge of the combined activity of these three agents in hog gastric mucin helps to explain the results recorded in the literature (Olitzki, 1948) dealing with the bacteriological use of mucin and its mode of action. The effect of the viscosity of the granules of 'whole' mucin (Macleod, 1941), and of the inorganic residue (Olitzki & Koch, 1945; Landy & Batson, 1949) have all been noticed, together with the inadequacy of each alone to account completely for the activity. The small virulence-enhancing activity which has been reported for many substances could be attributed, probably, to the possession of one or two but not all three of the factors contained in crude hog gastric mucin. A clearer picture of the mode of action of mucin should result from further bacteriological and physiological work in which the above relationship is recognized.

The purification of the third, more specific, factor involved in the enhancement of virulence is being continued using the revised biological assay described in this paper, in which an inactive suspension of a constant amount of particulate residue in a medium of constant viscosity is combined with the third factor. In this way only low concentrations of the latter factor are needed and the limited supply of fractionation samples is sufficient to inject adequate batches of mice. The mixture of charcoal and tragacanth was chosen after preliminary experiments had been carried out to find the maximum sensitivity of the assay for the virulence-enhancing samples. Charcoal is used as the particulate residue instead of the residue obtained from the hog gastric mucin, because the latter material is heavily contaminated with sporing organisms which are not destroyed by heating to 60° for 0·75 hr.; the charcoal suspension is sterile, or contains only one or two organisms in 0·2 ml.

It must be emphasized that there is no direct relationship between the activities of purified virulence-enhancing samples, and the activity of the original crude mucin. The former are measured in 'Virulence-enhancing units'/g. which refer to a new 'standard' preparation containing the third factor only, whereas the latter was measured in 'Wilson units' which refer to a crude 'standard' preparation containing all three of the factors involved in the virulence enhancement.

SUMMARY

1. Insoluble particulate matter and a viscous medium, alone and combined, enhance the virulence of *Bacterium typhosum* for mice.

2. The activity of hog gastric mucin is due to a synergic action between a particulate residue, a viscous medium and a more specific third factor.

3. The biological assay of samples of the third factor has been revised in the light of this knowledge. It can detect with probability, $P = 0{\cdot}95$, a difference in activity of two to two and a half times, using the weighted mean of duplicate assays.

It is a pleasure to record our gratitude to Dr D. W. Henderson for his helpful criticism.

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ADDENDUM

Statistical Analysis of the Biological Assay of Virulence-enhancing Activity

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About four tests are carried out weekly, each involving a comparison of the 'standard' preparation with unknown virulence-enhancing samples. The death rates of the 'standard' preparation in four to eight tests are investigated every week or fortnight for homogeneity of response in each dose group and for parallelism of the respective probit regression lines. Over such a short period of time a mean regression line for the 'standard' preparation should be a more reliable basis for comparison with the unknown sample than that of the individual 'standard' in the test and the fiducial limits are considerably narrower when using a 'mean standard'. The results quoted in Table 4 are obtained by this method. It may well happen that the three factors which should remain constant in the assay (viscosity, particulate matter and the inherent virulence of the *Bact. typhosum*) do in fact vary, so that the regression lines of the various standard tests are not parallel. In this case, an individual comparison with wider fiducial limits is made; such assays are regarded as pilot experiments only. The relative potencies thus obtained are expressed in units/g. ('Virulence-enhancing units') different from 'Wilson units' (Smith, 1950*a*), since the latter refer to a crude 'standard' preparation which contains all three factors affecting the virulence enhancement.

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Amino-acid Requirements of Amylase Synthesis by Pigeon-Pancreas Slices

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In previous publications (Hokin, 1950, 1951*a*), it was shown that pigeon-pancreas slices synthesize α-amylase when incubated in glucose saline media and that the addition of acid-hydrolysed casein supplemented with tryptophan, or a mixture of twenty amino-acids, increased the rate of synthesis approximately threefold.

The present paper is concerned with the role of the individual amino-acids in α-amylase synthesis. A mixture of ten amino-acids (tryptophan, arginine, threonine, valine, tyrosine, lysine, leucine, histidine, phenylalanine and isoleucine) was found to be necessary and also sufficient for the maximum rate of amylase synthesis.

Part of this work has been communicated to the Biochemical Society (Hokin, 1951*b*).

EXPERIMENTAL

Media. The medium used for the incubation of slices was the bicarbonate saline of Krebs & Henseleit (1932) gassed with 5% CO_2 in O_2. A similar saline containing only 0·00043 M-bicarbonate not gassed, was used as a suspending medium for slices before incubation. Glucose (0·2%) was added in all experiments.

Amino-acids. Commercial amino-acids were used. The standard mixtures of amino-acids and the final concentrations in the medium are shown in Table 1. In earlier experiments mixtures I and III, which contained L-amino-acids, with the exception of DL-serine and DL-threonine, were used. Mixture I contained the ten amino-acids found to be essential for the nutrition of the rat (Rose, 1938) and chick (Almquist, 1947), whilst mixture III contained ten 'non-essential' amino-acids. In the later experiments, after it had been established by paper chromatography that three of the L-amino-acids from natural sources; i.e. leucine, isoleucine and tryptophan were not pure, mixtures II and IV, which contained synthetic racemic amino-acids, with the exception of L-arginine, L-histidine and L-hydroxyproline, were used. Stock solutions of mixtures II and IV were made up by dissolving the amino-acids in water and neutralizing with 1 N-NaOH. Mixtures I and II were added from stock solutions in which the amino-acids were dissolved in bicarbonate saline.

Ketonic acids. Indolylpyruvic acid was synthesized from indole aldehyde according to the method of Ellinger & Matsuoka (1920). It was light buff coloured; it darkened at 200° and melted at 205° (uncorr.). Indole aldehyde was

Table 1. *Standard amino-acid mixtures*

(Concentrations are those in incubation vessels in routine experiments.)

Mixture I		Mixture II		Mixture III		Mixture IV	
Amino-acid	Concn. (M)	Amino-acid	Concn. (M)	Amino-acid	Concn. (M)	Amino-acid	Concn. (M)
L-Tryptophan	0·002	DL-Tryptophan	0·002	L-Glutamate	0·002	DL-Glutamate	0·002
L-Arginine	0·002	L-Arginine	0·001	L-Aspartate	0·002	DL-Aspartate	0·002
DL-Threonine	0·004	DL-Threonine	0·002	L-Alanine	0·002	DL-Alanine	0·002
L-Lysine	0·002	DL-Lysine	0·002	L-Glycine	0·002	Glycine	0·001
L-Leucine	0·002	DL-Leucine	0·002	DL-Serine	0·004	DL-Serine	0·002
L-Valine	0·002	DL-Valine	0·002	L-Proline	0·002	DL-Proline	0·002
L-Histidine	0·002	L-Histidine	0·001	L-Hydroxyproline	0·002	L-Hydroxyproline	0·001
L-Isoleucine	0·002	DL-Isoleucine	0·002	L-Tyrosine	0·002	DL-Citrulline	0·001
L-Phenylalanine	0·002	DL-Phenylalanine	0·002	L-Cystine	0·002	DL-Methionine	0·001
L-Methionine	0·002	DL-Tyrosine	0·002	L-Citrulline	0·002	L-Citrulline	0·002

synthesized from indole by the method of Boyd & Robson (1935). *p*-Hydroxyphenylpyruvic acid was obtained from Homburg Chemiewerk, Frankfurt a/M. Sodium α-keto *iso*caproate and sodium α-keto *iso*valerate were kindly given by Dr A. Meister, National Institutes of Health, U.S.A. Indolylpyruvic acid and *p*-hydroxyphenylpyruvic acid were neutralized with 0·16M-$NaHCO_3$.

Stock solutions of amino-acids and ketonic acids were stored in the frozen state at approximately −10°.

Preparation and incubation of tissues. In previous experiments (Hokin, 1951*a*) the slices were placed in a chilled humidified crystallizing dish before incubation. In the earlier experiments of the present series the slices were suspended in chilled saline as soon as they were cut, where they remained until a sufficient number of slices had been prepared. However, the results were less consistent than with the original procedure (Hokin, 1951*a*), which was therefore re-instituted. Further improvements which increased precision were the rejection of all surface slices and the use of at least 70 mg. of tissue per vessel, derived from the anterior (large) lobe only. These modifications limited the number of parallel incubations from the pancreas of one pigeon. All of the data, except those presented in Table 2, are from experiments in which this improved technique was used.

Slices were incubated at 40° in beakers of about 18 ml. capacity in the metabolic incubator of Dubnoff (1948). Periods of incubation were 2–3 hr.

Methods of assay. Amylase activity was assayed by the method of Smith & Roe (1949) with the following modifications. Instead of adding the various ingredients separately to the assay tube, the starch was dissolved in a buffered solution which had been heated to the boiling point in a water bath. This solution contained 0·056M-NaCl and 0·067M-phosphate buffer, pH 7·2. After cooling to room temperature 9 ml. of the buffered starch solution was pipetted into each assay tube. The final volume and concentrations of the constituents in the assay tube were as recommended in the original procedure of Smith & Roe (1949). When enzyme solutions were assayed by the original and modified methods the amylase activities agreed within experimental error.

Determination of total α-amino nitrogen of pancreas tissue. Freshly removed pigeon pancreas (about 100–200 mg. tissue per determination) was placed in about 8 vol. water in a boiling-water bath for 2 min. The tissue and boiled extract were transferred to an all-glass homogenizer (Potter & Elvehjem, 1936). After homogenizing for about 10 sec., 1 vol. 1N-H_2SO_4 and 1 vol. 10% sodium tungstate were added, and the homogenization was continued for about a minute. The homogenate was transferred to a graduated centrifuge tube, the volume adjusted to 6 ml. and the mixture was centrifuged. Total α-amino N was determined on 5 ml. portions by the ninhydrin method of Van Slyke, Dillon, MacFadyen & Hamilton (1941), as modified by Hamilton & Van Slyke (1943).

Calculations. In this paper the difference between the total amylase activity of incubated slices (see Hokin, 1951*a*) and the amylase activity of unincubated slices is referred to as 'amylase units synthesized'. The effect of omitting an amino-acid is expressed by the following quotient: Percentage reduction in synthesis = [('amylase units synthesized' in standard mixture) − ('amylase units synthesized' in deficient mixture)] × 100/('amylase units synthesized' in standard mixture). In experiments in which the early technique was followed (see above) only those experiments were used in which synthesis in the standard mixture during the period of incubation exceeded 50 amylase units/mg. dry wt. (range 56–186 units). As described previously (Hokin, 1951*a*), 1 unit of Smith & Roe (1949) is equivalent to 0·25 μg. crystalline pig α-amylase.

RESULTS

Amino-acid requirements for maximum amylase synthesis

When the mixture of the twenty amino-acids shown previously to stimulate amylase synthesis (Hokin, 1951*a*) was replaced by one containing only the ten 'essential' amino-acids there was no significant effect on the rate of amylase synthesis. The mean per cent difference between synthesis in the presence of mixtures I plus III and mixture I was 1% for five experiments with a range of −19 to +19% and an S.E.M. of 7·4.

On the other hand, Table 2 shows that when any one 'essential' amino-acid was omitted there was a statistically significant reduction in synthesis, except in the case of methionine.

Table 2. *Effect of omission of essential amino-acids on α-amylase synthesis*

(Bicarbonate saline; 0·2% glucose; standard mixtures I plus III in four experiments and I alone in the remainder of experiments; 40°; incubation period 2–3 hr.; slices suspended in saline before incubation.)

Amino-acid omitted	Reduction in synthesis (%) Mean	Range	S.E.M.	*P*	No. of experiments
L-Methionine	−4·9	+25 to −29	6·7	0·450	8
L-Phenylalanine	−13	+7 to −29	4·6	0·020	9
L-Isoleucine	−18	+15 to −46	6·4	0·020	10
L-Histidine	−22	−3 to −55	5·4	0·004	9
L-Valine	−29	−9 to −46	5·4	0·002	7
L-Leucine	−32	+6 to −58	9·2	0·012	6
L-Lysine	−42	−15 to −69	7·3	0·002	6
L-Threonine	−55	−43 to −74	3·2	<0·001	6
L-Arginine	−59	−39 to −66	4·7	<0·001	6
L-Tryptophan	−66	−50 to −75	4·2	<0·001	6
All	−60	−20 to −76	6·2	<0·001	8

Subsequently it was found by paper chromatography that certain samples of the L-amino-acids contained other amino-acids as impurities. The L-tryptophan (L. Light and Co. Ltd., Wraysbury, Bucks) contained tyrosine; the L-leucine (Light's) contained tyrosine, methionine and cystine. The L-isoleucine (F. Hoffman-La Roche and Co., Basel, Switzerland) contained valine. The amounts of contaminating amino-acids were judged to be no more than 1 %. The remaining natural amino-acids were pure. Experiments on the omission of the amino-acids which were present as contaminants were therefore repeated using mixtures made up predominantly of synthetic amino-acids (mixtures II and IV). With twenty amino-acids added the rate of amylase synthesis was the same either in the presence of predominantly DL-amino-acids or in the presence of L-amino-acids; thus, the D compounds were not inhibitory.

Results from experiments using the 'purer' mixtures (II and IV) are summarized in Table 3. The omission of tyrosine now produced a mean reduction in synthesis of 53 %. The small amounts of tyrosine present as contaminant in the experiments recorded in Table 2 were thus sufficient to meet the needs of maximum amylase synthesis under the conditions of these experiments. Whilst in 'impure' mixtures the 'omission' of valine had caused a mean reduction of 29 % (Table 2) its complete omission from mixtures II and IV produced a mean reduction of 67 %.

Eight 'non-essential' amino-acids plus DL-methionine (mixture IV) still had no statistically significant effect on synthesis when added to the nine remaining 'essential' amino-acids plus tyrosine (mixture II). Thus methionine, although essential for the rat (Rose, 1938), chick (Almquist, 1947) and human (Rose, Johnson & Haines, 1950) was not required under the conditions of these experiments. This is consistent with the recent observation that methionine is the only 'essential' amino-acid not present in crystalline α-amylase (Boissonnas, 1950). The addition of L-cystine to mixtures II plus IV produced in three experiments a mean reduction in synthesis of 12 % (range 10–15 %; S.E.M. 2·2 %), which is significant. This inhibition was not affected by the omission of serine or its addition in larger quantities indicating that the metabolism of serine was not inhibited competitively by cystine.

Table 3 also shows the results of experiments in which the omission of phenylalanine and isoleucine was studied by the improved technique described in the experimental section. Lack of phenylalanine or isoleucine produced a mean reduction of 21 and 15 % respectively, as compared to 13 and 18 % in the experiments recorded in Table 2. The higher degree of precision of this technique, as compared to the earlier one, is borne out by the far greater degree of statistical significance achieved in about half as many experiments.

It will be noted in Tables 2 and 3 that when either tryptophan, arginine, threonine or valine was omitted, synthesis was about the same as in the complete absence of added amino-acids.

Replacement of tryptophan, tyrosine, valine and leucine by their respective ketonic acids in the synthesis of α-amylase

In order to test whether certain amino-acids necessary for maximum synthesis can be replaced by their corresponding α-ketonic acids, indolylpyruvate, *p*-hydroxyphenylpyruvate, α-keto*iso*caproate and α-keto*iso*valerate were substituted for tryptophan, tyrosine, valine and leucine, respectively, as shown in Table 4. Addition of ketonic acids was found to be as effective, or almost as effective, as their respective amino-acids in supporting maximum amylase synthesis. This suggests that the ketonic acids were aminated, presumably by transamination. The recent work of Cammarata & Cohen (1950) indicates that transamination reactions in the tissues studied by these authors cover a wider range of amino-acids than was thought previously (Cohen & McGilvery, 1949).

Transamination in pigeon pancreas. To test whether certain 'non-essential' amino-acids can arise by transamination of intermediates of carbohydrate metabolism—homogenates of pigeon pancreas were incubated with α-ketoglutarate and various amino-acids. Considerable quantities of glutamate (determined by the method of Gale (1945) as modified by Krebs, 1948) were formed from alanine and aspartate. On the other hand, no significant quantities of glutamate were formed from

Table 3. *Omission of amino-acids from 'pure' amino-acid mixtures*

(Bicarbonate saline; 0·2 % glucose; standard amino-acid mixtures consisted of mixtures II plus IV; 40°; incubation period 3 hr.)

Amino-acid omitted	Reduction in synthesis (%) Mean	Range	S.E.M.	*P*	No. of experiments
Mixture IV	+2·8	+13 to −14	5·9	0·644	5
Isoleucine	−15	−5 to −27	3·4	0·007	6
Phenylalanine	−21	−14 to −26	2·1	<0·001	5
Tyrosine	−53	−34 to −71	8·2	<0·001	4
Valine	−67	−64 to −72	2·4	<0·001	3

Table 4. *Replacement of tryptophan, tyrosine, leucine and valine by their respective ketonic acids*

(Bicarbonate saline; 0·2% glucose; 40°; incubation period 3 hr.)

Exp. no.	Amino-acid omitted	Ketonic acid added	'Amylase units synthesised' (units of Smith & Roe (1949) per mg. dry wt.)
1	None	None	68*
	DL-Tryptophan	None	26
	DL-Tryptophan	Indolylpyruvic acid	62
2	None	None	71*
	DL-Tyrosine	None	52
	DL-Tyrosine	*p*-Hydroxyphenylpyruvic acid	69
3	None	None	83
	DL-Leucine	None	50
	DL-Leucine	α-Keto*iso*caproic acid	84
	DL-Valine	None	23
	DL-Valine	α-Keto*iso*valeric acid	87

* Mean of duplicate slices.

glycine, DL-serine, DL-tyrosine, DL-tryptophan, DL-phenylalanine, or DL-proline. These data indicate that aspartic acid and alanine can arise in pigeon pancreas by transamination of intermediates of the tricarboxylic acid cycle.

The failure of tryptophan and tyrosine and possibly other amino-acids to form significant quantities of glutamate is probably due to the relatively insensitive technique of detection used (see Cammerata & Cohen, 1950).

Total α-amino nitrogen of pigeon pancreas. The mean free α-amino nitrogen of the pancreas of four pigeons was 36 mg. N/100 g. tissue, range, 28–45, which is of the same order as the values found by Hamilton (1945) for various tissues of the dog.

DISCUSSION

Amino-acids and protein synthesis. In preliminary experiments Boissonnas (1950) has found by paper chromatography the following amino-acids in crystalline α-amylase in decreasing order of concentration: aspartic acid, alanine, glycine, leucine, and isoleucine, valine and serine, phenylalanine, glutamic acid, threonine, lysine, arginine, proline, histidine, tyrosine and tryptophan. It is of considerable interest that all of the 'essential' amino-acids and only one 'non-essential' amino-acid (tyrosine) are needed for maximum synthesis. The fact that tyrosine is necessary indicates that pigeon pancreas is unable to convert phenylalanine to tyrosine.

It is also of interest that methionine, which is the only 'essential' amino-acid not present in α-amylase (Boissonnas, 1950) is not needed for amylase synthesis.

A comparison of the total α-amino nitrogen of pigeon pancreas with the data of Krebs, Eggleston & Hems (1949) indicates that glutamic acid plus glutamine comprise about 50% of the total amino nitrogen of pigeon pancreas. These compounds, by transamination of intermediates of carbohydrate metabolism, may give rise to alanine and aspartic acid in sufficient quantities to satisfy the needs of amylase synthesis. The source of the remaining 'non-essential' amino-acids, i.e. glycine, serine and proline, which are present in α-amylase, but which are not needed for its synthesis under the conditions of these experiments is not known. These three amino-acids may be formed in pancreas from non-protein sources or they may exist in concentrations sufficiently high to satisfy the requirements of amylase synthesis. The data of Wiss (1949) indicate that glycine and serine, but not proline, exist in relatively high concentrations in liver. Preliminary experiments indicate that considerable proteolysis occurs during the incubation of pancreas slices. This would obviously be another source of amino-acids, but the data presented here indicate that this process cannot supply adequate amounts of 'essential' amino-acids.

Peters & Anfinsen (1950) have recently reported a net synthesis of serum albumin by chicken-liver slices, but no stimulation of albumin synthesis was observed when a mixture of amino-acids was added. The fact that amino-acids do not stimulate albumin synthesis in chicken-liver slices may be due to the fact that the rate of protein synthesis in this system is below that rate which is likely to be limited by the tissue stores of free amino-acids. It has also been observed by Borsook, Deasy, Haagen-Smit, Keighly & Lowy (1950) that the *in vitro* incorporation of labelled amino-acids into tissue proteins of guinea pig liver homogenate is not stimulated by the addition of mixtures of non-labelled amino-acids. Greenberg, Friedberg, Schulman & Winnick (1949), on the other hand, have observed a twofold stimulation of glycine-1 ^{14}C uptake by the addition of a mixture of unlabelled amino-acids to adult rat-liver

homogenates but not to homogenates of foetal liver. Later, Winnick (1950), by dialysing foetal-liver homogenates, obtained a stimulation of glycine-1 ^{14}C uptake by the addition of an unlabelled amino-acid mixture, and he suggested that the previous failure to obtain a stimulation of glycine-^{14}C uptake in foetal liver homogenates was probably due to relatively higher amino-acid concentrations prevailing in embryonic tissues.

The variable effects of added amino-acid mixtures on the *in vitro* synthesis of specific proteins and on the incorporation of labelled amino-acids into proteins may be accounted for by several factors. It is likely that the *in vitro* incorporation of labelled amino-acids into tissue proteins is a resultant of both net protein synthesis and a 'dynamic state' in which no additional protein is formed. The relative contribution of each of these factors to the total amino-acid turnover will depend on the particular system employed. The *in vitro* stimulation of either the synthesis of specific proteins or the incorporation of labelled amino-acids into proteins by added amino-acid mixtures would probably depend on the amount of new protein being formed and the concentrations of endogenous amino-acids.

SUMMARY

1. Of the sixteen amino-acids found in crystalline α-amylase (Boissonnas, 1950) ten have been found necessary and also sufficient for maximum synthesis of the enzyme by pigeon-pancreas slices. These amino-acids are: tryptophan, arginine, threonine, valine, tyrosine, lysine, leucine, histidine, isoleucine and phenylalanine. They include every 'essential' amino-acid present in crystalline α-amylase, and one 'non-essential' amino-acid, i.e. tyrosine.

2. In the synthesis of amylase, tryptophan, tyrosine, valine and leucine can be replaced 80–100 % by their respective ketonic acids.

3. The origin of the 'non-essential' amino-acids is discussed. Glutamate plus glutamine comprise about 50 % of the total free amino nitrogen of pigeon pancreas. These compounds by transamination of intermediates of carbohydrate metabolism, may give rise to aspartic acid and alanine.

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Studies in Detoxication

39. NITRO COMPOUNDS. (*a*) THE METABOLISM OF *o*-, *m*- AND *p*-NITROPHENOLS IN THE RABBIT. (*b*) THE GLUCURONIDES OF THE MONONITROPHENOLS AND OBSERVATIONS ON THE ANOMALOUS OPTICAL ROTATIONS OF TRIACETYL *β*-*o*-NITROPHENYLGLUCURONIDE AND ITS METHYL ESTER

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As a result of the discovery that nitro compounds occur in nature there is likely to be a renewed interest in their fate in the body. The antibiotic chloromycetin (chloramphenicol) has been shown to be a *p*-substituted nitrobenzene, and the plant glycosides, hiptagin and karakin, have been shown by Carter & McChesney (1949) to contain *β*-nitropropionic acid (hiptagenic acid).

It is possible that the toxicity and perhaps the physiological effects of nitro compounds are related to the modifications which the nitro group may undergo *in vivo*. The work of Channon, Mills & Williams (1944) on the metabolism of 2:4:6-trinitrotoluene (TNT) has shown that the aromatic nitro group may be reduced *in vivo* to a hydroxylamino and then to an amino group, and it appears that the biological reduction of the nitro group follows the path, $NO_2 \rightarrow NO \rightarrow NHOH \rightarrow NH_2$. Evidence in support of this path has been obtained by Bueding & Jolliffe (1946) who showed that reduced coenzyme I plus purified Straub flavoprotein probably converts TNT to a nitroso compound, that xanthine oxidase can convert TNT to the hydroxylamine and that whole tissue (rat liver and diaphragm) is necessary to convert TNT to the amine. In some compounds, however, the nitro group may be relatively resistant to reduction *in vivo*, for Glazko, Wolf, Dill & Bratton (1949) have reported that little if any chloromycetin is reduced in man (see also Glazko, Dill & Rebstock, 1950).

Our intention in the present work was to study the extent of reduction of the three mononitrophenols and attempt to relate it to their orientation and to the fact that *p*-nitrophenol is much more toxic than the *ortho*-compound. Beutner & Block (1943) and Dietrich & Beutner (1946) have shown that *o*-nitrophenol is relatively non-toxic and is largely excreted by rabbits with its nitro group unaltered. We also wished to isolate and characterize the glucuronides of the nitrophenols as a preliminary to a study on the metabolism of nitrobenzene. Furthermore, tetra-acetyl *β*-*o*-nitrophenyl-D-glucoside shows an anomalous positive optical rotation (Glaser & Wulwek, 1924; Montgomery, Richtmyer & Hudson, 1942), and we were desirous of finding out whether or not a similar rotation would be shown by the corresponding acetylated *β*-glucuronide.

METHODS

Materials. The *o*-, *m*- and *p*-nitrophenols used in this work were commercial products which were purified by recrystallization from ligroin or aqueous ethanol and had m.p. 45°, 96° and 140° respectively.

Determination of o-nitrophenol. This phenol can readily be determined colorimetrically using the yellow colour produced in alkaline solution. A straight-line calibration curve was obtained when the colour from 0·1 to 1 mg. of *o*-nitrophenol in 10 ml. 0·05 N-NaOH (0·1 or 5 N-NaOH can be used without affecting the colour) was measured in the Spekker absorptiometer using Ilford Spectrum blue filter no. 602. By this method, 0·1 mg. in 10 ml. solution could be estimated with an error of ±1%. For the estimation of free *o*-nitrophenol in urine, the phenol was steam-distilled out of the urine at about pH 6·5. Quantities of the order of 0·5–1·0 mg. of the phenol could be recovered quantitatively from 10 ml. of urine, if the volume of distillate were not less than 10 ml.; usually 90–100% were recovered in the first 10 ml.

Rabbits were given *o*-nitrophenol suspended in water, by stomach tube. The 24 hr. urine (10 ml., pH 6·5) was placed in a micro-Kjeldahl distillation apparatus. Steam was then passed through the apparatus until 10 ml. of distillate were collected. The free *o*-nitrophenol usually distils in the first few ml. and the operation is complete in 4–5 min. The distillate was then treated with 1 ml. 2 N-NaOH and the yellow colour measured in the Spekker absorptiometer. The results are given in Table 1, from which it can be concluded that nearly all the *o*-nitrophenol excreted is in a combined form.

Table 1. *Free o-nitrophenol in rabbit urine*

Rabbit no.	Dose of *o*-nitrophenol (g.)	Dose of *o*-nitrophenol (mg./kg.)	Free *o*-nitrophenol (mg.)	Free *o*-nitrophenol (% of dose)
29	0·6	200	1·74	0·29
32	0·6	200	3·12	0·52
33	0·6	200	8·22	1·37
9	1·0	450	13·0	1·3

Determination of total nitro groups with titanous chloride. A convenient amount of urine (1–5 ml.) containing the nitro

compound was boiled with 10 ml. of conc. HCl for 10 min. in a stream of CO_2 and then a measured excess amount of acid titanous chloride added. (The titanous chloride solution was prepared by boiling 50 ml. commercial (20%) $TiCl_3$ with 100 ml. conc. HCl for 1 min. and making up to 2 l. with water. The solution was kept in an inert atmosphere.) The mixture was kept boiling for a further 10 min. in the atmosphere of CO_2 and then the excess titanous chloride was estimated with standard ferric alum (0·3 M) using potassium thiocyanate as indicator near the end point. At the same time, a blank titration was performed on the titanous chloride solution omitting the urine. The difference between the two titrations gives the amount of reducible material in the urine calculated as nitro compound.

Samples of the three mononitrophenols (2–3 mg. in 10–20 ml. solution) could be recovered from pure solutions within 2% of the calculated values. The error in the recoveries of these phenols from urine was slightly greater than this, and normal urines contained reducible material equivalent to 0–10 mg. of nitrophenol per 100 ml. However, at the dose levels used in the present experiments, the error introduced by this material can be neglected. The titanous chloride reagent estimates all compounds of higher oxidation level than aminophenol, i.e. nitrophenol, nitrosophenol and hydroxylaminophenol. If appreciable amounts of the nitroso and hydroxylamino compounds are present, there will be a tendency to low values for nitro compound not reduced to amino, since the intermediates require only a fraction of the titanous chloride needed for an equivalent amount of nitro compound. As will be clear from the results (Table 2) the amounts of these intermediates appearing in the urine cannot be of much significance.

Determination of p-*aminophenol.* *p*-Aminophenol can readily be determined in quantities of 10–100 μg. in volumes up to 10 ml. by means of the indophenol reaction if $K_3Fe(CN)_6$ is used as the oxidizing agent. Hypochlorite, hypobromite, H_2O_2 and KSO_4 were tried as oxidizing agents, but were all found unsatisfactory. The procedure adopted was to take a suitable volume (up to 10 ml.) of *p*-aminophenol solution and make it alkaline with 5 ml. N-Na_2CO_3. Then 1 ml. of 5% (w/v) aqueous phenol was added followed by 5 ml. of 0·1% (w/v) aqueous $K_3Fe(CN)_6$. The volume was made up to 25 ml. with water, and after 30 min. the blue colour was measured in the Spekker absorptiometer using Ilford Spectrum orange filter no. 607. (In the presence of *p*-nitrophenol the solution is green in colour, but with the use of the filter the aminophenol can be estimated in the presence of nitrophenol.) The calibration curve was a straight line.

For the determination of *p*-aminophenol in *p*-nitrophenol urine rather more ferricyanide is necessary than in pure solution. A mixture of 1 ml. urine and 1 ml. conc. HCl was heated on a boiling-water bath for 2 hr. to hydrolyse the conjugated forms of the aminophenol. The solution was then diluted to 100 ml. with water. To 10 ml. of this diluted urine, was added 1 ml. 5% phenol, 5 ml. N-Na_2CO_3 and 8 ml. of 0·1% $K_3Fe(CN)_6$. After making up to 25 ml. and keeping 30 min. the colour was read in the Spekker absorptiometer as above. The recoveries of *p*-aminophenol from pure solutions of *p*-aminophenylglucuronide were 86–96% (average 93%). (See Table 2 for results.)

Determination of m-*aminophenol.* The determination of this phenol was carried out by diazotization and coupling with naphthylethylenediamine as follows (cf. Bratton & Marshall (1939) procedure for sulphanilamide) as follows: a solution of *m*-aminophenol (10 ml.) containing up to 250 μg. of the phenol was treated with 0·5 ml. 4 N-HCl and 1 ml. 0·1% (w/v) $NaNO_2$ solution. Diazotization was allowed to proceed for 10 min., after which time 1 ml. of 0·5% (w/v) ammonium sulphamate was added and the mixture allowed to stand 2 min. Then 1 ml. of a 1% aqueous solution of *N*-(1-naphthyl) ethylenediamine hydrochloride was added as coupling reagent and the red colour allowed to develop for 4 hr. After diluting with water to 25 ml. the colour intensity was read in the Spekker absorptiometer using an Ilford Spectrum yellow-green filter no. 605. The standard curve obtained was a straight line.

For the estimation of total *m*-aminophenol in *m*-nitrophenol urine, 0·5 ml. urine and 0·5 ml. of 4 N-HCl were heated in a boiling-water bath for 4 hr. (The *m*-aminophenol may be present in the urine as the glucuronide and ethereal sulphate and as *N*-acetyl conjugates. The ethereal sulphates are readily hydrolysed in about 15 min., but the glucuronides require prolonged heating for complete hydrolysis. Under the above conditions of hydrolysis, recoveries of *m*-aminophenol from its glucuronide at levels of 100–200 μg. were 96–106% (average 100%); high results are obtained if the hydrolysis is not quite complete, for the glucuronide gives more colour than the free phenol.) The hydrolysed urine was then diluted with water to 10 ml. and the procedure described in the preceding paragraph followed. (See Table 2 for results.)

Determination of o-*aminophenylglucuronide.* The *o*-aminophenol in *o*-nitrophenol urine is present as the glucuronide and the ethereal sulphate. The total amount of *o*-aminophenol in the urine is difficult to assess because *o*-aminophenylglucuronide is relatively resistant to acid hydrolysis (Hanson, Mills & Williams, 1944). The amount of *o*-aminophenylglucuronide in the urine, however, can readily be assessed by diazotization and coupling by the Bratton & Marshall procedure. In this procedure the *o*-aminophenol occurring as ethereal sulphate is not estimated since *o*-aminophenylsulphuric acid readily loses its sulphate group on diazotization (Burkhardt & Wood, 1929) to yield *o*-aminophenol which gives no colour with the Bratton & Marshall reagents. The extent of reduction of *o*-nitrophenol could therefore only be partly assessed.

The method used was as follows (Smith & Williams, 1949). The urine (0·5 ml.), 0·5 ml. 4 N-HCl and 3 ml. of water were mixed and heated in a test tube in a boiling-water bath for 1 hr. This procedure deacetylates any *o*-acetamidophenylglucuronide and hydrolyses ethereal sulphates. The solution was cooled diluted to 10 ml. and diazotized with 1 ml. of 0·1% $NaNO_2$ solution. The remainder of the method was as described above, except that the colour was allowed to develop for 24 hr. The amount of *o*-aminophenylglucuronide present was then read from a standard curve produced by subjecting known amounts of *o*-aminophenylglucuronide to the same treatment. In this way 10–60 μg. of *o*-aminophenol as its glucuronide could be readily estimated, and the recoveries were 100 ± 5%.

Glucuronic acid was determined by the method of Hanson *et al.* (1944).

Ethereal sulphates were estimated turbidimetrically according to Sperber (1948). Inorganic sulphate was removed from the urine with acid $BaCl_2$ solution, the excess of the latter being removed by Na_2CO_3 solution and centrifuging. Samples of the supernatant were hydrolysed, and the liberated sulphate estimated turbidimetrically after the addition of solid $BaCl_2.2H_2O$ using the Spekker absorptio-

Table 2. *Excretion of nitro compounds and amino derivatives after the feeding of* o-, m- *and* p-*nitrophenols to rabbits*

Nitrophenol	Rabbit no.	Dose (mg./kg.)	Amounts excreted (% of dose)		
			Unchanged nitro group*	Reduced to amino compound†	Total
Ortho	5	330	77·9	3·5	81·4
	7	330	83·1	1·8	84·9
	12	330	85·7	2·4	88·1
Meta	5	200	70·3	6·9	77·2
	7	200	68·0	8·0	76·0
	16	200	78·5	11·0	89·5
	17	200	86·2	9·2	95·4
	20	200	69·8	12·9	82·7
Para	15	200	88·5	10·8	99·3
	16	200	91·7	13·2	104·9
	17	200	82·0	18·5	100·5

* The excretion of nitro compounds was virtually complete in 1 day.
† Estimated for 2 days after dosing.

meter with Ilford Spectrum orange filter no. 607. The Spekker readings were referred to a standard curve prepared with potassium *p*-nitrophenylsulphate. The results of glucuronic acid and ethereal sulphate determinations are given in Table 3.

Table 3. *Glucuronic acid and ethereal sulphate conjugations of the mononitrophenols*

Nitrophenol	Rabbit no.	Dose (mg./kg.)	Glucuronide (% of dose)	Ethereal sulphate (% of dose)
Ortho	29	200	65·5	12·2
	35	200	76·0	7·3
	36	200	70·5	13·0
Meta	29	200	71·5	28·0
	36	150	82·5	16·0
	40	200	81·5	14·0
Para	29	200	59·0	12·5
	36	200	61·5	20·5
	40	150	74·0	15·0

Detection of the minor metabolites of the nitrophenols by paper chromatography and spectroscopy

Reference compounds. 3-Nitrocatechol (m.p. 86°) and 4-nitrocatechol (m.p. 168–170°) were prepared according to Weselsky & Benedikt (1882) and 2-nitroresorcinol (m.p. 85°) according to Kauffman & Pay (1904). Nitroquinol (m.p. 132°) was obtained by demethylation with concentrated HBr of nitroquinol dimethyl ether prepared according to Mühlhäuser (1881).

Paper chromatography. The above compounds and the mononitrophenols were readily separated by paper partition chromatography using benzene, acetic acid and water (1:1:2 by volume) and Barcham Green no. 401 paper. For the determination of R_F values (see Table 4) the compounds were applied to the paper (which had been equilibrated to the solvent vapour for 24 hr.) in ethereal solution, and the chromatogram run for 15 hr. at 20°. Under these conditions, *o*-nitrophenol and 2-nitroresorcinol volatilized and gave no spot on spraying the paper. If the chromatogram was run at 0°, however, *o*-nitrophenol could be kept on the paper and formed a spot near the solvent front, but since this phenol is readily identified in small amounts by steam distillation and spectroscopy, its chromatographic identification was not pursued at this point. For development of the spots on the paper, it was sprayed with N-NaOH, with which each phenol gives a distinctive colour (see Table 4). *p*-Nitrophenol and 4-nitroquinol, however, have the same R_F value, but in the nitrophenol urines examined, they could not be expected to appear together.

For more precise identification and approximate estimation of the dihydroxy compounds the spots on the unsprayed paper were cut out under illumination by ultraviolet light. In this light, the phenols quenched the weak background fluorescence of the paper and showed up as

Table 4. *Chromatography of the nitrophenols*

(Solvent: benzene, acetic acid, water (1:1:2, v/v). Chromatograms run for 15 hr.)

Compound	R_F value	Colour of spot with N-NaOH	Absorption spectrum in 0·05 N-NaOH of the phenol in the visible region	
			$\lambda_{max.}$ (mμ.)	$\epsilon_{max.}$
4-Nitrocatechol	0·21	Red	510	10,500
4-Nitrophenol	0·62	Canary yellow	—	—
Nitroquinol	0·62	Purple	540	3,900
3-Nitrophenol	0·65	Yellow	—	—
3-Nitrocatechol	0·68	Mauve	—	—

dark spots which could be cut out with minimum contamination from other materials from urine.

For spectroscopic identification, a chromatogram was prepared with a spot of urine extract running alongside the authentic phenols. Suitable small areas of the paper were cut out as small pointed strips (2·5 × 10 cm.) and the phenols eluted chromatographically into graduated flasks. The monohydric phenols were rapidly eluted with ethanol, whereas the dihydric phenols required 0·1 N-NaOH for rapid elution. The solutions of the dihydric phenols were then diluted with water so that the NaOH concentration was 0·05 N and the absorption spectra of the solutions in the 500 mμ. region determined in a Unicam spectrophotometer. The relevant data for the two dihydric phenols found are given in Table 4.

Feeding experiments. *p*-Nitrophenol (250 mg.) was fed in aqueous solution to a rabbit. The 24 hr. urine was treated with 0·1 vol. of conc. HCl and heated at 100° for 20 min. to hydrolyse ethereal sulphates. After cooling, the urine was continuously extracted for 6 hr. with ether. Aminophenols were removed from the extract with 2 N-HCl and the residual nitrophenols transferred to 2 N-NaOH. The latter was acidified and the nitrophenols transferred to 10 ml. ether which was then used for paper chromatography.

In *p*-nitrophenol urine only one dihydric phenol was found in the ethereal sulphate fraction, namely 4-nitrocatechol. The 4-nitrocatechol spot was cut out and eluted as above. In this way, it was found that roughly 0·1–0·2 % of the *p*-nitrophenol fed appeared in the ethereal sulphate fraction as 4-nitrocatechol.

Similar experiments were carried out with *o*- and *m*-nitrophenols. The former yielded small amounts of nitroquinol, (approx. 0·03 % of the dose), and the latter 4-nitrocatechol (approx. 0·03 %) and traces of nitroquinol (<0·002 %) as ethereal sulphates.

Elbs persulphate oxidation of the mononitrophenols

Baker & Brown (1948) have suggested that a free radical, the sulphate ion radical, is involved in the Elbs persulphate oxidation of phenols. The possibility that biological hydroxylation is a free radical process has been discussed by us (Smith & Williams, 1950; Smith, 1950), and it was interesting, therefore, to find out whether the Elbs persulphate oxidation of the mononitrophenols produced the ethereal sulphates of the same dihydric phenols as those found biologically. In the persulphate oxidation an ethereal sulphate group is introduced into the phenol by alkaline persulphate, and hydrolysis of the hydroxyphenylsulphate produced, yields a diphenol. In the present work the diphenols were not all isolated, but were detected chromatographically by the technique described above.

A mixture of the *o*-, *m*- or *p*-nitrophenol (2 g.), KOH (4 g.), KSO_4 (4 g.) and water (100 ml.) was allowed to stand at room temperature for 48 hr. A further 2 g. of the persulphate was added and the mixture left a further 24 hr. The mixture was then acidified at 0° and extracted with ether to remove unchanged nitrophenol. The aqueous residue was then heated under reflux at 100° for 40 min. to hydrolyse ethereal sulphates and after cooling the liberated phenols were extracted with ether. The ether extract was evaporated and the dihydric phenols present identified by paper chromatography using benzene, acetic acid and water (1:1:2) as described above. *o*-Nitrophenol yielded nitroquinol and traces of 3-nitrocatechol (both have been isolated by Forrest & Petrow, 1950); *m*-nitrophenol yielded mainly nitroquinol with appreciable amounts of 3- and 4-nitrocatechol; and *p*-nitrophenol yielded 4-nitrocatechol only (see Table 6). In large-scale experiments potassium 4-hydroxy-3-nitrophenylsulphate was easily isolated from the Elbs persulphate oxidation of *o*-nitrophenol, and potassium 4-hydroxy-2-nitrophenylsulphate from *m*-nitrophenol (unpublished observations).

The glucuronides of the mononitrophenols

(Melting points are uncorrected; $[\alpha]_D$ was measured in 2 dm. tubes and where $c=1$ the error is $\pm 1°$, and $c=0·1$, $\pm 5°$.)

o-Nitrophenol. A total of 12 g. of the phenol was fed in water by stomach tube to six rabbits. The 24 hr. urine was acidic (pH 6), gave a strong naphthoresorcinol test and a positive diazo test for aromatic amino compounds and was slightly reducing to Fehling's and Benedict's solutions and ammoniacal $AgNO_3$. The glucuronide fraction was isolated in the usual way with basic lead acetate. After removal of the lead with H_2S and then concentrating, a crude glucuronide gum was obtained which weighed 8·9 g. and contained much inorganic material. Extraction of this gum with 95 % ethanol yielded 2 g. of purified gum which would not crystallize. The gum (1·2 g.) was dissolved in 95 % methanol and added dropwise to an ethereal solution of diazomethane. After keeping an hour, the ether was evaporated leaving a neutral gummy methyl ester. The latter was acetylated with pyridine (5 ml.) and acetic anhydride (8 ml.) and the mixture kept overnight. The product was then poured into water whereupon the *triacetyl β-o-nitrophenylglucuronide methyl ester* crystallized (0·7 g.). A further yield of 1 g. of this ester was obtained from the ethanol-insoluble part of the glucuronide gum (total yield, 4·3 % of the dose). The ester was recrystallized from aqueous acetone and formed blunt needles, m.p. 172° and $[\alpha]_D^{22°}+18·5°$ ($c=2$ in $CHCl_3$). (Found: C, 50·7; H, 4·3; N, 3·3; OCH_3, 7·1; $COCH_3$, 28·1. $C_{19}H_{21}O_{12}N$ requires C, 50·1; H, 4·7; N, 3·1; OCH_3, 6·8; $COCH_3$, 28·4 %.)

The above triacetyl methyl ester (0·5 g.) was dissolved in 25 ml. ethanol and the solution saturated with NH_3 at 0°. The mixture was kept for 2 days at room temperature and then again saturated with gaseous NH_3. After keeping a further 24 hr., the solution was allowed to evaporate spontaneously and residue recrystallized from ethanol. The *β-o-nitrophenylglucuronidamide* separated very slowly from the ethanol as little nodules of small needles, m.p. 173–175° and $[\alpha]_D^{22°}-63·8°\pm 2°$ ($c=0·14$ in ethanol; 4 dm. tube). (Found: C, 45·9; H, 4·4; N, 8·7. $C_{12}H_{14}O_8N_2$ requires C, 45·9; H, 4·5; N, 8·9 %.)

Triacetyl β-o-nitrophenylglucuronic acid was prepared by dissolving 1 g. of the glucuronide gum in 5 ml. acetic anhydride and treating the mixture with 5 drops of perchloric acid (60 %). The mixture was kept for 0·5 hr. at room temperature and then carefully diluted with water. A buff precipitate separated which was filtered and washed with benzene. On recrystallization from aqueous ethanol, the *triacetyl acid* (0·54 g.) was obtained as buff needles, m.p. 195–196° and $[\alpha]_D^{20°}+31·2°$ ($c=1$ in $CHCl_3$). (Found: C, 48·9; H, 4·3; N, 3·3. $C_{18}H_{19}O_{12}N$ requires C, 49·0; H, 4·3; N, 3·2 %.)

m-Nitrophenol. From the glucuronide fraction of the urine of three rabbits which had each received 0·6 g. of *m*-nitrophenol there was isolated after acetylation and methylation of the glucuronide gum 0·5 g. (8·5% of dose) of *triacetyl β-m-nitrophenyl glucuronide methyl ester.* After recrystallization from absolute ethanol the compound formed needles, m.p. 159–160° and $[\alpha]_D^{20°} - 42·6°$ ($c = 0·8$ in $CHCl_3$). (Found: C, 50·7; H, 4·7; N, 3·4; OCH_3, 6·8. $C_{19}H_{21}O_{12}N$ requires C, 50·1; H, 4·7; N, 3·1; OCH_3, 6·8%.) The above ester (0·2 g.) was dissolved in 10 ml. methanol and the solution saturated with dry NH_3 at 0°. After keeping overnight in a stoppered flask and then concentrating, *β-m-nitrophenylglucuronidamide* (0·1 g. or 72%) was obtained as blunt needles. It was recrystallized from methanol containing a few drops of water and elementary analysis showed it to be a hemihydrate. It had m.p. 206° and showed $[\alpha]_D^{20°} - 91°$ ($c = 0·2$ in methanol). (Found: C, 44·7; H, 4·3; N, 8·6; $C_{12}H_{14}O_8N_2$. 0·5 H_2O requires C, 44·6; H, 4·4; N, 8·7%.)

p-Nitrophenol. Owing to its toxicity this phenol could only be fed at dose levels not exceeding 0·2 g./kg. As before, the glucuronide was isolated as *triacetyl β-p-nitrophenylglucuronide methyl ester* (0·5 g. from 1·2 g. of *p*-nitrophenol or 11·4% of dose). On recrystallizing from ethanol, the compound formed cigar-shaped crystals, m.p. 146° and $[\alpha]_D^{20°} - 51°$ ($c = 0·9$ in $CHCl_3$). (Found: C, 50·2; H, 4·6; N, 3·5; OCH_3, 6·7. $C_{19}H_{21}O_{12}N$ requires C, 50·1; H, 4·7; N, 3·1; OCH_3, 6·8%.) *β-p-Nitrophenylglucuronidamide* was obtained as above and formed needles from methanol (yield 75%); m.p. 210° and $[\alpha]_D^{20°} - 96°$ ($c = 0·17$ in methanol). (Found: C, 44·0; H, 5·0; N, 8·4. $C_{12}H_{14}O_8N_2 . H_2O$ requires C, 43·4; H, 4·9; N, 8·4%.)

The optical rotation of triacetyl β-o-nitrophenylglucuronide methyl ester

Tetra-acetyl *β-o*-nitrophenylglucoside has an abnormal positive rotation (Glaser & Wulwek, 1924; Montgomery *et al.* 1942) and Pigman (1944) showed that when the temperature was raised the rotation of the tetra-acetate in nitrobenzene became less positive and eventually became negative. The trend of the curve relating rotation to temperature suggested that at sufficiently high temperatures, the rotation would approach the value for the *m*- and *p*-isomers. It was suggested that the abnormal rotation was due to weak bonds between groups in the molecule and that at the higher temperature the vibrational energy of the molecules is greater and tends to disrupt these weak bonds. The following observations show that triacetyl *β-o*-nitrophenylglucuronide methyl ester behaves in a similar manner to the corresponding tetra-acetyl glucoside.

The specific rotation of a 0·63% solution of triacetyl *β-o*-nitrophenylglucuronide methyl ester in *cyclo*hexanone was measured in a 2 dm. jacketed polarimeter tube in a Bellingham and Stanley Model A polarimeter. The temperature of the water circulating through the tube was raised and readings of the rotation were taken at intervals of 10° from 20 to 90°. Six readings were taken at each temperature which varied by no more than 0·5° during each set of readings. The temperature was kept steady for several minutes before readings were taken to ensure uniform heating.

After reaching 90°, the solution was allowed to cool and readings again taken at intervals of 10°. No corrections were made for the expansion of the solution or polarimeter tube, since the error introduced by neglecting them is relatively small. The coefficient of cubical expansion of *cyclo*hexanone is 0·00091, and in our highest reading this would cause an error of 0·7%. Our results (Fig. 1) show that the rotation of triacetyl *β-o*-nitrophenylglucuronide methyl ester becomes

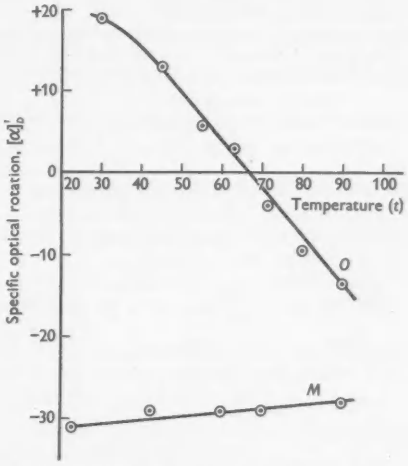


Fig. 1. The specific optical rotations at different temperatures of triacetyl *β*-nitrophenyl-D-glucuronide methyl esters in *cyclo*hexanone. *O*, *ortho*nitro compound; *M*, *meta*nitro compound.

less positive on raising the temperature and passes through zero at about 65°. In a similar way the effect of temperature on the rotation of a 0·432% solution of triacetyl *β-m*-nitrophenylglucuronide methyl ester in *cyclo*hexanone was studied (see Fig. 1). In this case there was little change in rotation between 23 and 92°.

DISCUSSION

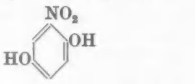
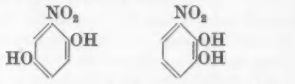
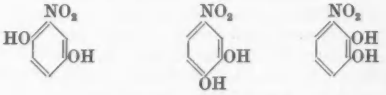
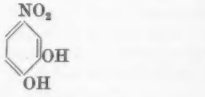
The early work of Meyer (1905) and Baumann & Herter (1877–8) had shown qualitatively that the *o*-, *m*- and *p*-nitrophenols conjugated *in vivo* with glucuronic and sulphuric acids. The present work shows that in doses of 0·2–0·3 g./kg. the conjugation is almost complete (Table 5), the major product in all three cases being the nitrophenyl glucuronide. Meyer (1905) was doubtful whether *o*-nitrophenol was reduced *in vivo*, although he was able to detect reduction of the *m*- and *p*-isomers. Table 5 shows that all three nitrophenols undergo reduction which is not extensive and which appears to be slightly greater for the *p*- than for the *m*- and *o*-isomers. *p*-Nitrophenol is much more toxic than its isomers (see Oettingen, 1949); *o*-nitrophenol, on the other hand, is a relatively innocuous substance (Beutner & Block, 1943). It seems unlikely, however, that

Table 5. *Metabolism of mononitrophenols—Summary*

	Percentage of dose excreted as					
	Nitro compounds	Amino compounds		Glucuronides	Ethereal sulphates	
Nitrophenol	*N*	*A*	$(N+A)$*	*G*	*E*	$(G+E)$*
ortho	82	3†	85	71	11	82
meta	74	10	84	78	19	98
para	87	14	101	65	16	81

* $(N+A)$ should be roughly equal to $(G+E)$, since the amounts of free phenols excreted were very small.
† This figure is low since only *o*-aminophenol combined with glucuronic acid was estimated. To allow for *o*-aminophenol combined with sulphuric acid, this figure could probably be doubled.

Table 6. *Orientation of biological hydroxylation and of Elbs persulphate oxidation of the mononitrophenols*

	Dihydric phenols* produced by	
Nitrophenol	Biological hydroxylation	Elbs persulphate oxidation
		
		
		

* The dihydric phenols are given in the quantitative order found experimentally.

the toxicity of *p*-nitrophenol can be explained in terms of the extent of its metabolic reduction, since this is not very much more than for its isomers. In any case, the product of the reduction, *p*-aminophenol, is not very toxic. However, the intermediates in the reduction, *p*-nitrosophenol and *p*-hydroxylaminophenol, may be more toxic, for Bueding & Jolliffe (1946) have shown, with TNT, that the intermediate products between the nitro compound and the amine inhibit respiration. We have observed that toxic doses of *p*-nitrophenol are very rapidly fatal, and this suggests that it is toxic *per se* and not as a result of metabolic changes. The latter very probably detoxicate *p*-nitrophenol.

The hydroxylation of the nitrophenols

Paper chromatography has shown that the nitrophenols are very slightly oxidized *in vivo* to dihydric nitrophenols. The extent of this oxidation, however, is less than 0·5% of the dose, and is unimportant from a practical point of view. The dihydric phenols produced are, however, interesting from the theoretical aspect, for the second hydroxyl group is introduced *o*- and/or *p*- to the existing hydroxyl group (cf. Smith, 1950), and its orientation is similar to that produced during the Elbs persulphate oxidation of the mononitrophenols (Table 6). Baker & Brown (1948) have suggested that the Elbs persulphate oxidation involves the sulphate ion radical, $\cdot OSO_3^-$. We have earlier suggested that the orientation of biological hydroxylation is similar to that found in free radical substitution (Smith & Williams, 1950; see also discussion by Stein & Weiss, 1950).

The anomalous optical rotation of β-o-nitrophenylglucuronide derivatives

The optical rotations of the derivatives of the β-nitrophenylglucuronides are given in Table 7. Both the triacetyl acid and triacetyl methyl ester of the *ortho*-derivative show anomalous positive rotations, whereas the corresponding *meta*- and *para*-isomers show the expected negative rotation characteristic of β-glycosides. The anomalous effect is obviously due to the presence of the three acetyl groups in the sugar ring combined with *ortho*-substitution of the aromatic ring by a positive group, for the effect disappears in the unacetylated glucuronide as shown by the normal negative rotation of β-*o*-nitrophenylglucuronidamide. The effect also tends to disappear on heating the solution, a point which has already been discussed. Abnormal positive rotations in the β-D-glucoside

series have also been observed with the tetracetates of *o*-chloromethyl-, *o*-bromomethyl-, *o*-iodomethyl-, 2:6-dinitro- and 2:4-dinitro- substituted β-D-phenylglucosides where the *ortho*-substituent is positive (Kunz, 1926; Pigman, 1944).

Table 7. *Optical rotations of β-nitrophenyl-D-glucuronide derivatives*

(For details of temperature and concentration, see text)

	Glucuronid-amide ($[\alpha]_D$ in methanol)	Triacetyl glucuronide methyl ester ($[\alpha]_D$ in $CHCl_3$)	Triacetyl glucuronic acid ($[\alpha]_D$ in $CHCl_3$)
β-*o*-Nitrophenyl	−63·8°	+18·5°	+31·2°
β-*m*-Nitrophenyl	−91°	−42·6°	—
β-*p*-Nitrophenyl	−96°	−51·0°	—

SUMMARY

1. A study has been made of the metabolism of the *o*-, *m*- and *p*-nitrophenols in the rabbit.

2. All three phenols are excreted in conjugated forms, there being only small amounts (< 1 %) of the unchanged free phenols excreted. The main metabolites are the nitrophenylglucuronides which account for about 70 % of the dose. The corresponding ethereal sulphates are also excreted.

3. The nitro group of the nitrophenols undergoes reduction *in vivo* only to a minor extent, and some 80 % of it is excreted unchanged. The extent of reduction is about 6 % for *o*-, 10 % for *m*- and 14 % for *p*-nitrophenol.

4. The nitrophenols also undergo oxidation to a very small extent (< 1 %); *o*- yields traces of nitroquinol, *m*-, nitroquinol and 4-nitrocatechol and *p*-, 4-nitrocatechol. The dihydric phenols produced are similar to those formed during the Elbs persulphate oxidation of the mononitrophenols. The significance of the similarity in relation to free radicals is discussed.

5. The paper chromatography of the mono- and di-hydric nitrophenols has been studied.

6. The β-glucuronides of the nitrophenols have been isolated and characterized as amides and triacetyl methyl esters.

7. The abnormal positive rotations of triacetyl β-*o*-nitrophenyl-D-glucuronide and its methyl ester have been investigated, and it has been shown that in the case of the ester this rotation becomes negative on raising the temperature, a behaviour reminiscent of tetra-acetyl β-*o*-nitrophenylglucoside.

8. The metabolic results have been discussed in relation to the toxicity of *p*-nitrophenol and the non-toxicity of *o*-nitrophenol.

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Studies in Detoxication

40. THE METABOLISM OF NITROBENZENE IN THE RABBIT. *o*-, *m*- AND *p*-NITROPHENOLS, *o*-, *m*- AND *p*-AMINOPHENOLS AND 4-NITROCATECHOL AS METABOLITES OF NITROBENZENE

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Despite its relatively highly toxic nature, nitrobenzene has been used under the name of 'oil of mirbane' or 'artificial oil of bitter almonds' for flavouring and perfuming purposes. Its main industrial use is in the manufacture of aniline and benzidine. In spite of the importance of nitrobenzene, little is known about its metabolism. Reports on the nature of the urine from men or animals which had received nitrobenzene always refer to the dark colour of the urine. This was attributed to the presence of *p*-aminophenol (Meyer, 1905) which was, up to the present, the only proved metabolite of nitrobenzene. Small amounts of nitrobenzene are also excreted unchanged (e.g. Frossard, 1925; Meyer, 1905). The mechanism of the conversion of nitrobenzene to *p*-aminophenol is still unknown, although much has been written about this and its relation to methaemoglobin formation (e.g. Dresbach & Chandler, 1918; Heubner & Meier, 1923; Lipschitz, 1920). In yeast, nitrobenzene is reduced to aniline, probably via nitrosobenzene and *N*-phenylhydroxylamine (Neuberg & Welde, 1914).

In the present work we shall show that in the rabbit, nitrobenzene gives rise to at least seven phenols as well as to small amounts of aniline. Although we have attempted a quantitative assessment of the metabolism of nitrobenzene, we feel that our results in this direction are not entirely satisfactory, and although the quantitative data enable us to make some interesting suggestions, further work is necessary.

METHODS

The effect of nitrobenzene on rabbits. Female giant chinchilla rabbits were used in this work and were kept on a diet of 70 g. rat cubes per day and water *ad lib.* The nitrobenzene was administered in 25 ml. water by stomach tube. The resistance of the rabbits to nitrobenzene varied greatly, since some resisted a dose of 370 mg./kg., whereas others were killed by 180 mg./kg. The highest level which could be fed with reasonable safety was 200 mg./kg. Above this level 50% of the animals died. The toxic effect was not immediately apparent, for with doses of the order of 250 mg./kg. death occurred between 2 and 6 days later. With doses above 300 mg./kg. death occurred within 24 hr.

Nitro groups. Nitro groups were estimated by the titanous chloride method described in the preceding paper (Robinson, Smith & Williams, 1951).

Total p-*aminophenol* was estimated by the indophenol method (Robinson *et al.* 1951).

p-*Aminophenylsulphate.* The *p*-aminophenol present as ethereal sulphate was estimated by making the urine 0·5 N with respect to HCl and heating at 100° for 10 min. This short hydrolysis decomposes the ethereal sulphate and the liberated *p*-aminophenol can then be estimated by the indo-

Table 1. *Nitro compounds and* p-*aminophenol derivatives in the urine of rabbits 2 days after receiving* 0·5 *g. nitrobenzene orally*

(Values bracketed represent: 1st day / 2nd day } Total of 1st and 2nd day.)

	Percentage of the dose excreted as						
					p-Aminophenylglucuronide		
Rabbit no.	Nitro compounds *N*	Total *p*-aminophenol *A*	*N* + *A*	*p*-Aminophenyl sulphate *B*	By difference (*A* − *B*)	By diazo reaction *C*	(*A* − *B*) − *C**
26	17·9 / 8·4 } 26·3	30·9 / 11·1 } 42	68	4·3 / 2·6 } 6·9	35	20·5 / 8·0 } 28·5	6·5
27	11·5 / 6·3 } 17·8	15·9 / 11·0 } 26·9	45	3·3 / 1·9 } 5·2	21·7	10·8 / 9·5 } 20·3	1·4
28	8·9 / 6·3 } 15·2	25·0 / 9·9 } 34·9	50	3·3 / 1·1 } 4·4	30·5	12·4 / 5·6 } 18·0	12·5

* This figure gives an estimate of *p*-acetamidophenylglucuronide.

phenol method. The difference between the total *p*-aminophenol and the *p*-aminophenylsulphate estimations should give approximately the amount of *p*-aminophenol combined with glucuronic acid. This procedure, however, does not allow for the presence of *p*-acetamidophenylsulphate which may or may not be present. Mild hydrolysis of this compound would probably remove the sulphate group but not necessarily the acetyl group.

p-Aminophenylglucuronide. The procedure used was the same as that used for the estimation of *o*-aminophenylglucuronide in the preceding paper (Robinson *et al.* 1951) except that the standard curve was constructed with *p*-aminophenylglucuronide instead of the *o*-isomer. The results of this estimation should roughly give the same values as the difference between the two preceding indophenol estimations. In general, however, the results should be, and are, a little lower since *p*-acetamidophenylglucuronide is not estimated by the diazo method and is almost certain to be present (see Table 1). The approximate agreement between the two methods of assessing *p*-aminophenylglucuronide also suggests that *o*- and *m*-aminophenylglucuronides, although almost certain to be present (since the phenols themselves have been isolated), are only present in small amounts compared with the *p*-isomer. This view is supported by the isolation experiments (see later, p. 231).

Mercapturic acid. The iodine consumption of nitrobenzene urine after alkaline hydrolysis was investigated as described by Parke & Williams (1951) for benzene urine. It was assumed that if nitrophenylmercapturic acid occurred in the urine it would be hydrolysed by alkali to nitrothiophenol. *o*- and *p*-Nitrothiophenols were prepared and shown to be stable to the alkali and the temperature used in the iodometric estimation of mercapturic acids. By iodine titration the percentage of nitrobenzene (at 150 mg./kg.) which may have been excreted as mercapturic acid was in three experiments 3·5, 2·8 and 4·8 %. These results are so low as to be just within the margin of error of the method and may be of no significance.

Detection and isolation of metabolites

Nature of nitrobenzene urine. Urine voided during the first 4 hr. after nitrobenzene administration was a clear golden-brown and slightly acid in reaction (pH 6·5). It gave a positive naphthoresorcinol reaction and turned a deeper yellow on adding alkali, showing the presence of nitro compounds. This urine gave only a feeble diazo reaction (nitrous acid and dimethyl-α-naphthylamine) before and after hydrolysis. It very slightly reduced Fehling's and Benedict's reagents. Ammoniacal silver nitrate was slowly reduced.

Urine voided 1 or 2 days after dosing was dark brown in colour and alkaline (pH 8). It gave an intense blue diazo reaction which became more intense after mild acid hydrolysis after which treatment it gave an intense indophenol reaction. After 2 days nitrophenols were not detectable, these being mainly excreted on the first day after dosing, with a little on the second. Most of the aminophenols had also been excreted on the first 2 days, although about 1 % of the dose still appeared as *p*-aminophenol on the third day. The urine, however, gave a weak diazo test for at least 7 days after dosing, suggesting that a part of the dose was being eliminated slowly. This is presumably the nitrobenzene which had been deposited in fatty tissues.

Steam-volatile metabolites of nitrobenzene

o-Nitrophenol. A total of 1·5 g. of nitrobenzene (A.R., b.p. 209°) was fed to three rabbits and urine was collected for 2 days. The urine was made 2N with respect to H_2SO_4, heated under reflux for 2 hr. on a boiling-water bath and then steam distilled. The first few ml. were bright yellow and contained *o*-nitrophenol. The yellow colour of the distillate was intensified on making alkaline, the colour being discharged on acidification.

The *o*-nitrophenol in the distillate was identified by paper chromatography and spectroscopy as follows. The absorption spectrum of the distillate was determined at acid and alkaline pH, and whilst all the maxima of *o*-nitrophenol (at 283 and 415 mμ. in 0·05N-NaOH and 280 and 351 mμ. in 0·5N-HCl) were present their extinctions were not in the correct ratios due to irrelevant absorption. The impurities present were shown to be, by colour tests, volatile phenols of normal urine.

In another experiment it was shown that phenol and *o*-nitrophenol could be separated by paper partition chromatography using benzene, *n*-butanol and NH_4OH (sp.gr. 0·880) in the ratio 5:2:2, as solvent. In this mixture, *o*-nitrophenol moves very slowly and if the chromatogram is run for 24 hr. no trace of phenol could be found on the paper, whereas *o*-nitrophenol had only moved a short distance.

Purification of the *o*-nitrophenol distillate for spectroscopy was carried out by partition chromatography on a silica column. The column (4 × 50 cm.), was packed dry with silica (Harrington's 'pure precipitated') containing an equal weight of the aqueous phase of the solvent mixture described above. The *o*-nitrophenol distillate (containing about 0·5 mg. of phenols) from nitrobenzene urine was extracted with ether, and the extract mixed with a little dry silica which was then placed on the top of the column of silica. The column was then developed with the benzene-butanol-ammonia mixture. After 4 hr. the column was allowed to drain, thus removing interfering phenols. The column was extruded and the yellow *o*-nitrophenol band cut out and suspended in 0·1N-NaOH and then steam distilled to remove ammonia and excess solvents. The suspension was then made acid and the *o*-nitrophenol present steam distilled. The steam distillate now showed the correct spectrum of *o*-nitrophenol in acid and in alkali with respect to the wavelengths of the maxima and their extinction coefficients (see Table 2). In this way, *o*-nitrophenol was satisfactorily identified in the urine.

For the estimation of *o*-nitrophenol, the urine of rabbits which had been fed 200–250 mg./kg. of nitrobenzene was hydrolysed as above and steam distilled until the distillate no longer had a yellow colour. The distillate was made up to 50 ml. with 0·1N-NaOH and the extinction of the solution at 415 mμ. measured in the Unicam spectrophotometer. Results are given in Table 3.

Nitrobenzene and aniline. A rabbit was fed 500 mg. of nitrobenzene by stomach tube and a 48 hr. urine was made alkaline (pH 9–10) with Na_2CO_3 and steam distilled. Under these conditions *o*-nitrophenol was not distilled and the

Table 2. *Spectroscopic data for o-nitrophenol, aniline and nitrobenzene*

		Authentic material			Urinary material	
Compound	Solvent	$\lambda_{max.}$	$\epsilon_{max.}$	$\epsilon_{max.\ short}/\epsilon_{max.\ long}$	$\lambda_{max.}$	E_{short}/E_{long}
o-Nitrophenol	0·05 N-HCl	280	6100	—	280	—
		351	3000	2·03	351	2·13
	0·05 N-NaOH	283	3850	—	283	—
		415	4500	0·86	415	1·04
Nitrobenzene	Water	270	7700	—	270	—
Aniline	0·1 N-NaOH	280	1500	—	280	—
	0·1 N-HCl	254	160	—	Maximum too small to be accurately measured	—

distillate contained nitrobenzene and aniline. That both these substances were present was suggested by the diazo test which was positive on the distillate and was increased in intensity when the distillate was reduced with Zn dust and HCl. To separate nitrobenzene, the distillate was made acid to congo red and steam distilled. This second distillate gave no diazo test until reduced with Zn dust and HCl. The absorption spectrum in water was determined, and it showed a large single band with a peak at 270 mμ. similar to that found for authentic nitrobenzene (cf. Doub & Vandenbelt, 1947). From the height of the peak and the known $\epsilon_{max.}$ (which was found to be 7700) for nitrobenzene, it was calculated that in 2 days after dosing 0·029 % of the nitrobenzene was excreted unchanged.

Table 3. *Excretion of o-nitrophenol by rabbits during 2 days after receiving nitrobenzene*

Rabbit no.	Weight (kg.)	Dose of nitrobenzene (mg.)	*o*-Nitrophenol excreted (% of dose)*
31	2·5	500	0·054
35	2·6	500	0·037
36	3·0	750	0·057
36†	3·0	750	0·165

* Estimated spectrophotometrically at 415 mμ. in 0·05 N-NaOH (see Table 2).

† Second dose fed to rabbit no. 36, 3 days after first dose.

The residue from the second steam distillation contained the aniline, and this was confirmed by distillation from alkaline solution and spectroscopic examination. In 0·1 N-NaOH a peak was found at 280 mμ. which disappeared on making acid. Aniline shows a peak at 280 mμ. ($\epsilon_{max.}$ 1500) in NaOH which disappears on acidification (see Table 2).

From the height of the peaks of the absorption spectrum it was calculated that in 2 days 0·5 % of the nitrobenzene fed appeared in the urine as aniline. This was confirmed in another experiment by colorimetric estimation of the aniline according to Smith & Williams (1949). By this method, the aniline excreted in 2 days was found to be 0·47 % of the dose of nitrobenzene.

The steam-volatile metabolites of nitrobenzene (0·5 g./rabbit) account for about 0·6–0·7 % of the dose in 2 days and consist largely of aniline (0·5 %) and traces of *o*-nitrophenol (0·05 %) and unchanged nitrobenzene (0·03 %).

Nitrobenzene in rabbit fat. A 3 kg. rabbit was given 0·75 g. of nitrobenzene by stomach tube with water. The animal died 2 days later. The fat (33 g.) around the kidneys and along the dorsal wall of the abdominal cavity was cut out and homogenized with 50 ml. water. The neutral homogenate was then steam distilled until 100 ml. of distillate had been obtained. This distillate gave no diazo reaction until reduced with Zn and HCl. On spectroscopic examination a peak at 270 mμ. was detected corresponding to nitrobenzene. From the extinction of the peak it was calculated that the above fat contained 0·54 % of the dose as unchanged nitrobenzene 2 days after dosing.

Nitrophenols

m- *and* p-*Nitrophenols and 4-nitrocatechol. Detection by paper chromatography.* The urine of a rabbit which had been fed 0·5 g. of nitrobenzene was collected for 36 hr. after feeding and made 4 N with respect to H_2SO_4. The mixture was heated under reflux for 4 hr. at 100°. After cooling, the solution was continuously extracted with ether for 6 hr. The extract (100 ml.) was washed once with dilute HCl and the nitrophenols transferred to 100 ml. 10 % NaOH. The alkaline solution was now acidified and the nitrophenols re-extracted with 100 ml. ether. This purified ether extract was now dried over anhydrous $CaCl_2$ and then used for paper chromatography.

For the paper chromatography of this solution, the paper and solvents used were those described in the preceding paper on nitrophenols (Robinson *et al.* 1951). Identification and elution for spectroscopic examination was also carried out as described before. The nitrophenols identified were *m*-nitrophenol (4 % of the dose), *p*-nitrophenol (5 %) and 4-nitrocatechol (0·5 %). *o*-Nitrophenol, is, of course, volatile and would not be detected by this procedure (Robinson *et al.* 1951).

Aminophenols

o-, m- *and* p-*Aminophenols. Detection by paper chromatography.* In our first experiments, benzene-*n*-butanol-water mixtures were tried, but it was found that in this solvent mixture *o*- and *m*-aminophenols had the same R_F values. Good separation was eventually obtained using wet *iso*propyl ether and Barcham-Green no. 401 paper. The solvent was prepared by shaking 2 l. of *iso*propyl ether with 200 ml. water, allowing the mixture to stand overnight and using the top layer. Owing to the volatility of the solvent, the tank had to be made carefully air-tight but it had the advantage

of rapid equilibration. This solvent travels rapidly and a good separation of the aminophenols can be obtained in as little as 6 hr. The R_F values obtained are given in Table 4. All the aminophenols can be located by spraying with ammoniacal silver nitrate which is reduced by these phenols which then show up as dark spots. A better method of spotting, however, was found in the use of a 1 % solution of salicylaldehyde in ethanol containing 5 % glacial acetic acid as spraying agent. The paper was sprayed with the reagent and, after drying at room temperature, the aminophenols show up as yellow spots which fluoresce a vivid yellow or green in ultraviolet light.

Table 4. *R_F values of aminophenols in wet* iso*propyl ether*

(Chromatograms run for 6 hr.)

Phenol	R_F
o-Aminophenol	0·77
m-Aminophenol	0·60
p-Aminophenol	0·40
4-Aminocatechol	0·065
4-Aminoresorcinol	0·10
2-Aminoresorcinol	0·41

The urine was hydrolysed and the nitrophenols extracted as already described (p. 230). The residual urine was then brought to pH 7–7·5 with solid $NaHCO_3$, a little sodium bisulphite added and the solution extracted continuously with peroxide-free ether for 24 hr. Large amounts of *p*-aminophenol (20–30 % of the dose) separated in the extract at this stage and were filtered off. The extracted aminophenols were then transferred to dilute HCl. The acid solution was neutralized with $NaHCO_3$ and the aminophenols transferred to peroxide-free ether, and this solution used for paper chromatography. In this way, the *o*-, *m*- and *p*-aminophenols were detected and their presence confirmed by spectroscopy. *o*-Aminophenol was eluted from its spot with ethanol and its spectrum in ethanol, acid and alkali determined. The maxima found ($\lambda_{max.}$ in ethanol, 288 and 232 mμ; in 0·1 N-HCl, 270 mμ.; and in 0·1 N-NaOH, 241 and 298 mμ.) were identical with those of authentic *o*-aminophenol. From the extinctions of the maxima, it was found that 0·54 % of the nitrobenzene fed was excreted as *o*-aminophenol. *m*-Aminophenol was eluted from its spot with 0·1 N-HCl and its amount was estimated both spectroscopically and with the Bratton and Marshall reagents as described above for aniline. By spectra in ethanol, the yield was 0·58 % of the dose and by the colorimetric procedure using diazotization followed by coupling with naphthylethylene diamine (Robinson *et al.* 1951), 0·3 %.

Isolation of the phenols of nitrobenzene metabolism

Nitrophenols. The 48 hr. urine from ten rabbits which had collectively received 6 g. of nitrobenzene was made 4 N with respect to H_2SO_4 and heated under reflux for 4 hr. at 100°. The hydrolysate was continuously extracted with ether for 18 hr. The ether was removed leaving a residue of mixed nitrophenols (0·25 g.). A large column (200 × 5 cm.) was packed with silica with which had been intimately mixed an equal weight of the aqueous phase of a solvent mixture of benzene-acetic acid-water (1:1:2). The phenols were intimately mixed with a little silica and this was placed at the top of the column as a layer 1–2 cm. thick. The column was then developed for 24 hr. with the non-aqueous layer of the solvent mixture. As soon as the eluates turned yellow on addition of alkali, collection of fractions was commenced. A single fraction (*A*) was then collected until the eluate, after reduction with Zn dust and HCl, showed the indophenol reaction. This fraction contained mainly *m*-nitrophenol. A second fraction (*B*) was then collected by allowing the solvent to run through until no further yellow colour was obtained on adding alkali.

Isolation of m-*nitrophenol.* Paper chromatography of fraction *A* showed that it contained mainly *m*-nitrophenol with small amounts of *p*-nitrophenol. The nitrophenols in *A* were extracted with 10 % NaOH, the extract acidified and the phenols taken into ether (3 × 50 ml.). The ether was evaporated. The solid residue was treated with 2–3 ml. of 10 % NaOH drop by drop. A small amount of the sparingly soluble Na salt of *p*-nitrophenol was filtered off and the filtrate acidified and extracted with ether. Evaporation of the ether left 65 mg. of *m*-nitrophenol which on benzoylation yielded 120 mg. of *m*-nitrophenyl benzoate, m.p. 94° and mixed m.p. 95° with authentic material, m.p. 95°. (Found: C, 64·1; H, 3·7. Calc. for $C_{13}H_9O_4N$: C, 64·2; H, 3·7 %.)

Isolation of p-*nitrophenol.* Fraction *B* was shown by paper chromatography to contain mainly *p*-nitrophenol with a little *m*-nitrophenol. The nitrophenols were isolated as in the preceding paragraph. The sparingly soluble Na salt of *p*-nitrophenol was filtered off and dissolved in acid and the acid solution ether extracted. Evaporation of the ether gave *p*-nitrophenol (30 mg. or 0·44 % of the dose), m.p. and mixed m.p. 114° after recrystallization from light petroleum. (Found: C, 51·5; H, 3·5. Calc. for $C_6H_5NO_3$: C, 51·8; H, 3·6 %.) The benzoate has m.p. and mixed m.p. 142°. (Found: C, 63·7; H, 3·6 %. Calc. for $C_{13}H_9O_4N$: C, 64·2; H, 3·7 %.)

Isolation of 4-*nitrocatechol.* The silica was extruded from the column and a greenish-yellow band near the top was cut out. The material in this band was extracted quickly with 10 % NaOH and the red extract centrifuged. It was then acidified and extracted with ether. The ether extract was dried with anhydrous $CaCl_2$ and evaporated to yield 20 mg. (0·26 % of the dose) of 4-nitrocatechol. Benzoylation by benzoyl chloride and alkali yielded 4-nitrocatechol dibenzoate, m.p. and mixed m.p. 156°, after two recrystallizations from aqueous ethanol. (Found: C, 65·9; H, 3·8. Calc. for $C_{20}H_{13}O_6N$: C, 66·1; H, 3·6 %.) In another experiment in which 4 g. of nitrobenzene were fed, the yields of benzoates were *m*-nitrophenol 70 mg., *p*-nitrophenol 70 mg. and 4-nitrocatechol 15 mg. In yet another experiment in which the nitrophenol fraction was not chromatographed and in which 6 g. of nitrobenzene were fed, only *m*-nitrophenol was isolated, in this case as the toluene-*p*-sulphonate (28 mg.) m.p. 113° and mixed m.p. 114°. (Found: C, 53·4; H, 3·8; Calc. for $C_{12}H_{11}O_5NS$: C, 53·2; H, 3·8 %.)

Aminophenols

p-*Aminophenol.* The urine from six rabbits which had collectively received 6 g. of nitrobenzene was hydrolysed and the nitrophenols removed as above. The urine was brought to pH 7·5–8·0 with solid $NaHCO_3$ and continuously extracted with ether for 6 hr. Evaporation of the ether yielded 0·54 g. (10 % of the dose) of almost pure *p*-aminophenol, m.p. 180° and mixed m.p. 181° (authentic m.p. 184°). The *ON*-dibenzoyl derivative was prepared and had m.p. and mixed m.p. 235°.

o-Aminophenol. The ether residues after crystallizing out the *p*-aminophenol as above was chromatographed on a column of silica (75 × 4 cm.) containing an equal volume of water. The column was developed with wet peroxide-free ether and 50 ml. fractions were collected. Each fraction was chromatographed on paper to establish its composition. The third fraction collected appeared to contain only *o*-aminophenol. It was evaporated and the light-brown residue was benzoylated with benzoyl chloride and pyridine. This yielded 100 mg. (0·64 % of the dose) of *ON*-dibenzoyl *o*-aminophenol. On recrystallizing twice from ethanol (charcoal) and once from ligroin (b.p. 120°), 20 mg. of the pure dibenzoyl derivative, m.p. and mixed m.p. 182° were obtained. (Found: C, 75·0; H, 4·9. Calc. for $C_{20}H_{15}O_3N$: C, 75·7; H, 4·8 %.) We were not successful in obtaining *m*-aminophenol or a derivative in a pure crystalline state.

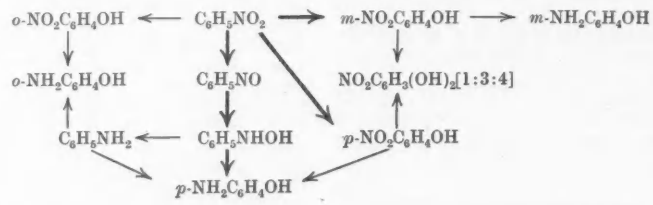
DISCUSSION

The present work has shown that the metabolism of nitrobenzene is complicated like that of aniline (Smith & Williams, 1949). At best we have only accounted for just over 50 % of the dose as the metabolites excreted during the 2 days subsequent to dosing. It appears that nitrobenzene is to some extent retained in the body of the rabbit for some time, for its metabolites can be detected in the urine a week after dosing. Our quantitative findings are briefly summarized in Table 5. It is clear that unlike the nitrophenols, nitrobenzene is quite extensively reduced *in vivo*, roughly two-thirds of the material excreted being in the reduced form and only one-third as nitro compounds. Furthermore, the amount of nitrobenzene excreted unchanged is almost negligible (0·03 %), so that the whole of the dose is either oxidized or reduced. The major metabolic product is *p*-aminophenol, which is not excreted in the free state but combined with glucuronic and sulphuric acids. The exact nature of these conjugates has not yet been investigated.

Table 5. *A summary of some of the quantitative aspects of the metabolism of nitrobenzene in the rabbit*

	Compounds found	Amounts excreted in 2 days (% of dose)	
Nitro compounds	Nitrobenzene	0·03	
	o-Nitrophenol	0·05	
	m-Nitrophenol	4·0	9·6
	p-Nitrophenol	5·0	
	4-Nitrocatechol	0·5	
	Total nitro compounds (by $TiCl_4$ titration)		20
Amino compounds	Aniline	0·50	
	o-Aminophenol	0·54	
	m-Aminophenol	0·58	36·2
	p-Aminophenol	35·0	

The *o*- and *m*-aminophenols are also present in small but appreciable amounts, and the *p*:*m*:*o* ratio is about 70:1:1. The fact that the *m*- and *p*-nitrophenols occur in roughly equal amounts, whereas *p*-aminophenol occurs in very much greater amounts than *m*-aminophenol, suggests that the major portion of the *p*-aminophenol is not derived from *p*-nitrophenol. Furthermore, since *p*-aminophenol is also present in much greater amounts than *o*-aminophenol, it suggests that *p*-aminophenol is not mainly derived from aniline, for although aniline does yield in the rabbit somewhat more *p*- than *o*-aminophenol, it does not do so in very great excess (Smith & Williams, 1949). Aniline, when fed to rabbits, gives rise to a highly reducing urine (Smith & Williams, 1949), the reducing substance being glucuronic acid which may be present



Quantitatively the metabolites of next importance are the *m*- and *p*-nitrophenols, which probably account for the major part of the nitro-compounds excreted. *o*-Nitrophenol is a relatively minor metabolite and using the figures in Table 5, the *p*:*m*:*o* ratio for the nitrophenols is 100:80:1. The numerical value of this ratio is given with reserve, but the *m*-and *p*-nitrophenols are certainly excreted in approximately equal amounts and in very much greater amounts than *o*-nitrophenol. The dihydric phenol, 4-nitrocatechol, is also present in appreciable amounts, there being roughly ten times as much of this phenol as *o*-nitrophenol.

as such or as a labile glucuronide. Nitrobenzene urine, however, is not highly reducing, and this suggests that aniline is not formed in large amounts. It seems, therefore, that *p*-aminophenol does not arise via aniline or *p*-nitrophenol. That a hydroxylamine is an intermediate in the biological reduction of nitro compounds seems to be established (Channon, Mills & Williams, 1944) and our present results suggest that most of the *p*-aminophenol may

be derived from nitrobenzene via phenylhydroxylamine. There is as yet no rigid proof (cf. Sieberg, 1914) that phenylhydroxylamine can be converted *in vivo* directly to *p*-aminophenol, although this change readily occurs *in vitro* in acid solution.

Minor sources of *p*-aminophenol are aniline, which is derived via phenylhydroxylamine, and *p*-nitrophenol. The formation of aniline has been proved by its spectroscopic detection, and *p*-nitrophenol is slightly reduced (14%) *in vivo* to *p*-aminophenol (Robinson *et al.* 1951). The probable main source of *o*-aminophenol is aniline although a very minute amount could arise from *o*-nitrophenol. The main source of *m*-aminophenol is probably *m*-nitrophenol, for aniline does not give rise to *m*-aminophenol *in vivo* (Smith & Williams, 1951, unpublished observations). The above scheme is suggested by our results (major metabolic paths are in thick arrows).

The metabolism of nitrobenzene and the orientation of biological hydroxylation

That *meta*-hydroxylation takes place *in vivo* was shown by Smith & Williams (1950) when they isolated *m*-cyanophenol as a metabolite of cyanobenzene. The isolation of *m*-nitrophenol as a metabolite of nitrobenzene suggests that *meta*-hydroxylation may be a general metabolic reaction for monosubstituted benzenes containing a *meta*-directing (ring deactivating) group.

Since nitrobenzene has now been shown to be hydroxylated in the rabbit in all three positions, *o*-, *m*- and *p*-, the process is therefore analogous with the hydroxylation of nitrobenzene by free hydroxyl radicals (Loebl, Stein & Weiss, 1950; Stein & Weiss, 1950). Similar results have been obtained with phenol which *in vivo* yields catechol and quinol but no resorcinol (Garton & Williams, 1949). The same two dihydric phenols are produced *in vitro* by the action of free hydroxyl radicals on phenol (Stein & Weiss, 1950) and, furthermore, the relative amounts of the two phenols produced are dependent upon pH. These results strongly suggest that the biological process may be free-radical in nature. There are, however, differences between the *in vivo* and *in vitro* results which, nevertheless, need not be contrary to the free-radical concept. In the first place, nitrobenzene is converted *in vivo* to 4-nitrocatechol, whereas *in vitro* the formation of this compound was not reported by Loebl *et al.* (1950). Loebl, Stein & Weiss (1951) have, in fact, commented upon the absence of dihydric phenols during the free-radical hydroxylation of benzoic acid which yields *o*-, *m*- and *p*-hydroxybenzoic acids. In the second place, *in vitro* free-radical hydroxylation of nitrobenzene produces *o*-, *m*- and *p*-nitrophenols in roughly equal amounts (Loebl *et al.* 1950), whereas *in vivo* the *m*- and *p*-isomers are produced in roughly equal amounts but in very much greater amounts than the *o*-isomer. This may mean that *in vivo* if a free radical is involved it may be very large and cannot readily approach the *ortho*-position.

The alternative explanation for the *in vivo* orientations is to assume the occurrence of a known metabolic reaction, namely 'perhydroxylation', which has been proved to occur to chlorobenzene (Smith, Spencer & Williams, 1950) and the polycyclic hydrocarbons (see Boyland, 1950; Young, 1950). If it be assumed that nitrobenzene forms two dihydrodiols in unequal amounts, there being much more of the 3:4 than the 2:3-diol, our results (and those with phenol) can be explained as follows. The 3:4-dihydrodiol on dehydration (see Fig. 1) could yield

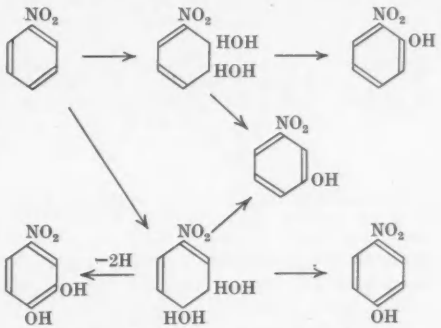


Fig. 1. Possible courses of perhydroxylation of nitrobenzene.

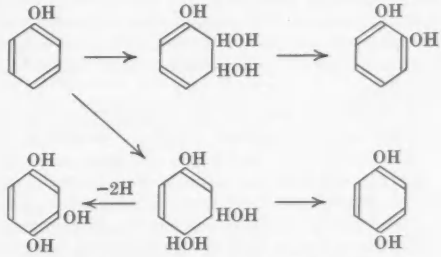


Fig. 2. Possible courses of 'perhydroxylation' of phenol.

m- and *p*-nitrophenol and on dehydrogenation, 4-nitrocatechol. The 2:3-dihydrodiol could yield *o*- and *m*-nitrophenol and 3-nitrocatechol. If it is assumed that only very small amounts of the 2:3-diol are formed, then the yield of *o*-nitrophenol would be very small and the 3-nitrocatechol almost undetectable. The *m*- and *p*-nitrophenols should be formed in roughly equal amounts (cf. cyanobenzene, Smith & Williams, 1950).

In the case of phenol the situation would be as shown in Fig. 2.

The dehydration of the dihydrodiols of phenol to give only one phenol and the dihydrodiols of nitrobenzene to two phenols can be explained, theoretically, by a consideration of the transition states in the dehydration. The dehydration is visualized as taking place as shown in Fig. 3. Whether or not the dihydrodiol (I) decomposes to give III or V will be determined by the speed of the two reactions which in turn will be dependent on the resonance energy of the two possible ionic transition states (II and IV in square brackets). When the group *X* is one of the classical *meta*-directing groups (e.g. NO_2 in nitrobenzene), no extra resonance stabilization is conferred on either of the ionic transition states (cf.

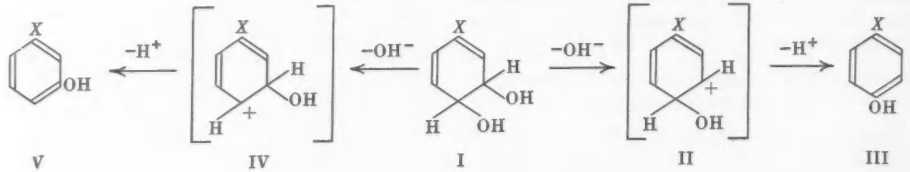


Fig. 3. Dehydration of dihydrodiols.

Dewar, 1949) and we may expect both reactions to proceed with comparable velocities to yield a mixture of the *m*- and *p*-phenols. On the other hand, if *X* is an *o*-*p*-directing group (e.g. OH in phenol) extra resonance stabilization is possible in the transition state (II) on the right and the dihydrodiol will yield the *p*-hydroxy compound III rather than the *m*-hydroxy compound V. An example of the latter case is 3:4-dihydro-chlorobenzene-3:4-diol which we have shown to dehydrate to *p*-chlorophenol only (Smith *et al.* 1950).

It appears to us that on our present evidence biological hydroxylation could be explained by direct hydroxylation by free radicals or by the 'perhydroxylation' hypothesis. The 'perhydroxylation' hypothesis is perhaps to be the more favoured at present. The whole process is, however, complicated by the fact that the phenols produced are conjugated and it is not absolutely certain whether conjugation is a secondary reaction to hydroxylation, although the conjugation of phenols is well known.

SUMMARY

1. A study has been made of the fate of nitrobenzene in the rabbit.

2. Nitrobenzene undergoes extensive reduction *in vivo* and at dose levels of 150–200 mg./kg. about 55 % is excreted as metabolites in the urine during 2 days after feeding; 20 % is in the form of nitro compounds and 35 % as amino compounds mainly *p*-aminophenol derivatives. Small amounts of nitrobenzene metabolites are excreted for a week after dosing, and appreciable amounts of nitrobenzene can be detected in the fat of the animal 2 days after dosing.

3. The nitro compounds found in the urine were nitrobenzene and *o*-nitrophenol, which occurred in very small amounts, *m*- and *p*-nitrophenol, which occurred in relatively large amounts, and 4-nitrocatechol. The last three phenols were isolated by partition chromatography on silica.

4. The amino compounds found were aniline, *o*- and *m*-aminophenol each amounting to about 0·5 % of the dose, and *p*-aminophenol which was the main metabolite (35 %). The *o*- and *p*-aminophenols were isolated.

5. Paper chromatography of the aminophenols has been studied using wet *iso*propyl ether as solvent.

6. All the phenols excreted were conjugated.

7. The results suggest that the main path of nitrobenzene metabolism is through phenylhydroxylamine to *p*-aminophenol. This has been discussed.

8. The results have also been discussed in relation to the perhydroxylation and free radical hypotheses of aromatic hydroxylation *in vivo*.

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Studies in Detoxication

41. A STUDY OF THE OPTICAL ROTATIONS OF THE AMIDES AND TRIACETYL METHYL ESTERS OF SOME BIOSYNTHETIC SUBSTITUTED PHENYLGLUCURONIDES

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Smith (1949) observed that the triacetyl methyl ester of β-*o*-cyanophenylglucuronide had an optical rotation in chloroform which was appreciably more negative than the corresponding *m*- and *p*-isomers. We decided, therefore, to prepare, by feeding the necessary phenols to rabbits, a number of such substituted phenylglucuronides and their derivatives to obtain further information on this point. Our results suggest that in the case of the triacetyl methyl esters of the *ortho*-substituted phenylglucuronides we have instances of the phenomenon of restricted rotation which shows itself as an abnormal negative optical rotation. The abnormal positive optical rotations of triacetyl β-*o*-nitrophenyl-D-glucuronide and its methyl ester have already been commented upon (Robinson, Smith & Williams, 1951).

EXPERIMENTAL

General method of preparation of glucuronides

In most cases the glucuronides were isolated as basic Pb salts. Two rabbits (3 kg.) were each given by stomach tube 2 g. or less of the phenol suspended in water. The 24 hr. urine was brought to pH about 4 with a little glacial acetic acid and then treated with saturated aqueous normal lead acetate until precipitation was complete. The precipitate was removed by centrifuging and then discarded. The supernatant fluid was now brought to pH about 8 with a little NH_3 (sp.gr. 0·88) and saturated aqueous basic lead acetate added in excess. The basic lead precipitate was washed on the centrifuge, made into a fine suspension in water and the lead removed by saturation with H_2S. After removal of PbS by filtration, the aqueous solution of the glucuronide was concentrated to a small volume *in vacuo* at 45°. Some glucuronides crystallize out at this stage, whereas others are obtained as water-soluble gums often containing inorganic material. These gums can be purified by dissolution in water and precipitation of the inorganic material by adding ethanol. The precipitate of inorganic material may sometimes contain salts of the glucuronides from which the glucuronide can be freed by careful addition of dilute H_2SO_4 to an ethanolic suspension. The extent to which these salts are formed appears to depend to some extent on the purity of the basic lead acetate used.

Methyl esters of glucuronides were prepared by dissolving the glucuronide gum in methanol and methylating with diazomethane in ether until the gum obtained on evaporating the solvents was neutral. In most instances the methyl esters were not obtained crystalline even when pure crystalline glucuronides were methylated. The methyl esters of glucuronides were found to be somewhat unstable and tended to decompose on boiling in water or drying at 100°.

Crystalline derivatives were usually obtained by acetylating the methyl esters at room temperature overnight with equal volumes of pyridine and acetic anhydride. On pouring the mixture into water, the triacetyl methyl ester of the glucuronide usually separated in crystalline form. Since these triacetyl esters crystallize easily, they are eminently suitable for characterizing glucuronides.

Crystalline glucuronidamides were prepared by dissolving the triacetyl methyl esters in methanol and saturating the solution with dry ammonia. The amides separated from the solution either on keeping or on concentrating. Most of these amides were sparingly soluble compounds of high melting point at which they decompose. Many tend to retain half a molecule of water. In view of their unsatisfactory melting points and sparing solubility, they are not nearly as suitable as the triacetyl methyl esters for characterizing glucuronides.

Melting points are uncorrected; rotations were measured in 2 dm. tubes and when *c* was about 1 %, the error in $[\alpha]_D$ was $\pm 1°$ or less. With the sparingly soluble glucuronidamides *c* was usually 0·1 and the error in $[\alpha]_D$ was $\pm 5°$. Compounds were dried at room temperature over $CaCl_2$, unless otherwise stated.

Fluorophenylglucuronides

o-*Derivatives.* Two rabbits each receiving 1 g. of *o*-fluorophenol (b.p. 151–152°/760 mm.) yielded from a 24 hr. urine, 2·7 g. of alcohol-soluble glucuronide gum. On methylation the latter yielded a non-crystalline methyl ester which was acetylated during 20 min. with acetic anhydride (7 ml.) and perchloric acid (2 drops, 60%). The mixture was diluted with water and *triacetyl β-o-fluorophenylglucuronide methyl ester* (2·6 g.) separated and was recrystallized from ethanol. It had m.p. 151–152° and $[\alpha]_D^{23°} - 44·7°$ ($c = 1$ in $CHCl_3$). (Found: C, 53·3; H, 5·0. $C_{19}H_{21}O_{10}F$ requires C, 53·3; H, 4·9%.) On treatment with methanolic ammonia *β-o-fluorophenylglucuronidamide*, m.p. 208–210° (decomp.) from ethanol and $[\alpha]_D^{20°} - 71°$ ($c = 0·1$ in methanol) was obtained. (Found: C, 50·1; H, 5·0; N, 4·4. $C_{12}H_{14}O_6NF$ requires C, 50·2; H, 4·9; N, 4·9%.)

p-*Derivatives.* The glucuronide gum from the 24 hr. urine of two rabbits which had each received 1 g. of *p*-fluorophenol (b.p. 185°/760 mm.) was prepared. After methylation, the product was acetylated with pyridine and acetic anhydride and yielded *triacetyl β-p-fluorophenylglucuronide methyl ester* (1·4 g.). On recrystallization from ethanol, it formed needles, m.p. 135° and $[\alpha]_D^{25°} - 33°$ ($c = 1$ in $CHCl_3$). (Found: C, 53·0; H, 5·1%.) *β-p-Fluorophenylglucuronidamide* was prepared as above and had m.p. 224° (decomp.) and $[\alpha]_D^{20°} - 77°$ ($c = 0·1$ in methanol). (Found: C, 49·8; H, 5·0; N, 4·5%.)

Chlorophenylglucuronides

o-*Derivatives.* *o*-Chlorophenol was fed to three rabbits (dose 1·5 g.) and the 24 hr. urine made acid to congo red with HCl. It was then continuously extracted with ether for 7 hr. Evaporation of the ether left a glucuronide gum (0·8 g.) which on methylation and acetylation yielded *triacetyl β-o-chlorophenylglucuronide methyl ester* (0·9 g.). This formed colourless needles from ethanol, m.p. 151–152° and $[\alpha]_D^{20°} - 65°$ ($c = 1$ in $CHCl_3$). (Found: C, 51·8; H, 4·9; OMe, 7·2. $C_{19}H_{21}O_{10}Cl$ requires C, 51·3; H, 4·8. OMe, 7·0%.)

p-*Derivatives.* Methylation of *p*-chlorophenylglucuronide (Spencer & Williams, 1950) yielded the *methyl ester* as an oil which crystallized at 0°. On recrystallization from water it formed needles, m.p. 157° and $[\alpha]_D^{26°} - 83°$ ($c = 0·5$ in water). (Found: OMe, 11·4. $C_{13}H_{15}O_7Cl$ requires OMe, 10·3%.) On acetylation the ester yielded *triacetyl β-p-chlorophenylglucuronide methyl ester* (needles from aqueous ethanol), m.p. 151–152° and $[\alpha]_D^{24°} - 32·9°$ ($c = 1·26$ in $CHCl_3$). (Found: C, 51·5; H, 4·5; OMe, 6·6. $C_{19}H_{21}O_{10}Cl$ requires C, 51·3; H, 4·8; OMe, 7·0%.) Acetylation of *p*-chlorophenylglucuronide (0·5 g.) with acetic anhydride (1 ml.) and a trace of perchloric acid, yielded, on dilution of the acetylation mixture with water, *triacetyl β-p-chlorophenylglucuronic acid* (0·6 g.). This formed leaflets from aqueous methanol, m.p. 175–176° and $[\alpha]_D^{20°} - 20·8°$ ($c = 1·2$ in $CHCl_3$). (Found: C, 50·2; H, 4·4. $C_{18}H_{19}O_{10}Cl$ requires C, 50·2; H, 4·4%.)

Bromophenylglucuronides

o-*Derivatives.* The glucuronide gum from the 24 hr. urine of two rabbits which had received 2 g. each of *o*-bromophenol (b.p. 195–198° at 760 mm.) was methylated and acetylated. It yielded 2·2 g. *triacetyl β-o-bromophenylglucuronide methyl ester* as colourless needles, m.p. 141–143° (from ethanol) and $[\alpha]_D^{20°} - 64·6°$ ($c = 1$ in $CHCl_3$). (Found: C, 47·0; H, 4·1; CMe, 6·2. $C_{19}H_{21}O_{10}Br$ requires C, 46·6; H, 4·3; OMe, 6·3%.)

β-o-Bromophenylglucuronidamide formed needles from water, m.p. 202–205° (decomp.) and $[\alpha]_D^{20°} - 63·4°$ ($c = 0·4$ in water). (Found: C, 40·4; H, 4·2; N, 3·8; Br, 21·4. $C_{12}H_{14}O_6NBr$, $0·5H_2O$ requires C, 40·4; H, 4·2; N, 3·9; Br, 22·4%.)

m-*Derivatives.* From 2·8 g. of *m*-bromophenol (b.p. 264–266° at 760 mm.) fed to two rabbits, there was similarly obtained 2·1 g. of *triacetyl β-m-bromophenylglucuronide methyl ester*, as needles from ethanol, m.p. 121° and $[\alpha]_D^{20°} - 33·1°$ ($c = 1$ in $CHCl_3$). (Found: C, 46·9; H, 4·4; OMe, 6·1%.) *β-m-Bromophenylglucuronidamide* formed colourless needles of the monohydrate from water, m.p. 215–218° (decomp.) and $[\alpha]_D^{20°} - 57°$ ($c = 0·2$ in water). (Found: C, 39·6; H, 4·5; N, 4·1; Br, 21·1. $C_{12}H_{14}O_6NBr$, H_2O requires C, 39·4; H, 4·4; N, 3·8; Br, 21·8%.)

p-*Derivatives.* A total of 3·5 g. of *p*-bromophenol was fed to two rabbits and the urine was worked up after 4 hr. The glucuronide gum in this case crystallized and 1·2 g. of *β-p-bromophenylglucuronide* was obtained as colourless needles (from water) of the *dihydrate*, m.p. 157° and $[\alpha]_D^{20°} - 68·2°$ ($c = 1$ in water). (Found: C, 37·5; H, 4·4; Br, 19·7. $C_{12}H_{13}O_7Br$, $2H_2O$ requires C, 37·4; H, 4·4; Br, 20·7%.) From the urine of the next 20 hr., the glucuronide gum was prepared, methylated and acetylated to give 1·2 g. of *triacetyl β-p-bromophenylglucuronide methyl ester* (needles from ethanol) m.p. 157–158° and $[\alpha]_D^{20°} - 28°$ ($c = 1$ in $CHCl_3$). (Found: C, 47·0; H, 4·5; OMe, 6·1%.) *β-p-Bromophenylglucuronidamide* formed colourless needles from water, m.p. 255–265° (decomp.) and $[\alpha]_D^{20°} - 61°$ ($c = 0·05$ in water). (Found: C, 41·3; H, 4·0; N, 4·7. $C_{12}H_{14}O_6NBr$ requires C, 41·4; H, 4·1; N, 4·0%.)

Iodophenylglucuronides

o-*Derivatives.* After feeding 6 g. *o*-iodophenol (m.p. 43°) 4·71 g. of purified glucuronide gum was obtained. The gum (1 g.) in a little water was neutralized with the calculated amount of Na_2CO_3, and on evaporation *sodium β-o-iodophenylglucuronate* was obtained as white needles with $[\alpha]_D^{20°} - 59°$ ($c = 1$ in water). (Found: C, 34·6; H, 3·2; I, 32·7; Na, 5·5. $C_{12}H_{12}O_7INa$ requires C, 34·5; H, 2·9; I, 30·4; Na, 5·5%.) The rest of the gum was converted into the gummy methyl ester (2·22 g.) which on acetylation yielded 1·9 g. of *triacetyl β-o-iodophenylglucuronide methyl ester*, m.p. 161–162° (from methanol) and $[\alpha]_D^{20°} - 63·3°$ ($c = 1$ in $CHCl_3$). (Found: C, 42·7; H, 4·3; $C_{19}H_{21}O_{10}I$ requires C, 42·6; H, 4·0%.)

p-*Derivatives.* From the urine of a rabbit which had received 2 g. of *p*-iodophenol (m.p. 92°) there was isolated 1·76 g. of *p-iodophenylglucuronide sesquihydrate.* It formed white needles from water, m.p. 154° and $[\alpha]_D^{20°} - 68·8°$ ($c = 0·4$ in water). (Found: C, 34·1; H, 3·8; I, 30·0. $C_{12}H_{13}O_7I$, $1·5\ H_2O$ requires C, 34·1; H, 3·8; I, 29·3%.) On methylation with ethereal diazomethane *p-iodophenylglucuronide methyl ester* was obtained which crystallized with difficulty from water. The ester melted at about 158°; $[\alpha]_D^{20°} - 68°$ ($c = 1$ in ethanol). (Found: C, 37·0; H, 4·1; OMe, 7·5. $C_{13}H_{15}O_7I$, $0·5\ H_2O$ requires C, 37·2; H, 3·6; OMe 7·4%.) On acetylation it yielded *triacetyl β-p-iodophenylglucuronide methyl ester*, m.p. 167–168° (from methanol) and $[\alpha]_D^{20°} - 25·8°$ ($c = 1$ in $CHCl_3$). (Found: C, 42·5; H, 4·2; OMe, 6·2. $C_{19}H_{21}O_{10}I$ requires C, 42·6; H, 4·0; OMe, 5·8%.)

Cresylglucuronides

o-*Derivatives.* Four rabbits received 1 g. each of *o*-cresol and an 18 hr. urine yielded 6·1 g. of crude glucuronide gum. After methylation and acetylation 0·64 g. of pure *triacetyl β-o-cresylglucuronide methyl ester* was obtained as white needles (from ethanol) m.p. 131° and $[\alpha]_D^{20°}$ −46·7° (*c* = 1 in $CHCl_3$). (Found: C, 56·7; H, 5·7; OMe, 7·2. $C_{20}H_{24}O_{10}$ requires C, 56·6; H, 5·7; OMe, 7·3%.) The residues from the preparation of the triacetyl methyl ester on treatment with ammonia yielded 0·13 g. of *β-o-cresylglucuronidamide*, m.p. 200–202° (from methanol) and $[\alpha]_D^{20°}$ −71·2° (*c* = 0·1 in ethanol). (Found: C, 54·1; H, 6·2; N, 4·8. $C_{13}H_{17}O_6N$, 0·5 H_2O requires C, 53·3; H, 6·2; N, 4·8%.)

m-*Derivatives.* Three rabbits receiving 1 g. each of *m*-cresol yielded 5 g. of glucuronide gum. Half of this gum on methylation and acetylation yielded 1 g. of *triacetyl β-m-cresylglucuronide methyl ester*, m.p. 93–94° (from ligroin) and $[\alpha]_D^{21°}$ −25·6° (*c* = 1 in $CHCl_3$). (Found: C, 56·7; H, 5·8; OMe, 7·4%.) The mother liquors from the acetyl ester treated with NH_3 yielded *β-m-cresylglucuronidamide*, m.p. 222–224° (from ethanol-ether) and $[\alpha]_D^{20°}$ −68·7° (*c* = 0·4 in ethanol). (Found: C, 52·7; H, 6·2; N, 4·8%.)

p-*Derivatives.* Five rabbits each receiving 0·5 g. *p*-cresol yielded 4·36 g. of crude glucuronide gum, 3·5 g. of which yielded 0·73 g. of *triacetyl β-p-cresylglucuronide methyl ester*, m.p. 140° and $[\alpha]_D^{20°}$ −36° (*c* = 1 in $CHCl_3$). (Found: C, 56·7; H, 5·8; OMe, 7·3%.) *β-p-Cresylglucuronidamide* formed white needles, m.p. 257–259° from methanol-water with $[\alpha]_D^{23°}$ −76·6° (*c* = 0·1 in methanol). (Found: C, 52·2; H, 6·1; N, 4·7. $C_{13}H_{17}O_6N$, H_2O requires C, 51·8; H, 6·4; N, 4·7%.)

Phenylphenylglucuronides (diphenylylglucuronides)

o-*Derivatives.* Four rabbits were each fed with 2·5 g. of the sodium salt of *o*-hydroxydiphenyl (British Drug Houses Ltd.) and a 6 hr. urine collected. The urine was acidified to congo red and extracted continuously with ether for 16 hr. The extract was evaporated to a syrup which was dissolved in water, and freed from unchanged *o*-hydroxydiphenyl by shaking with light petroleum. The residue was evaporated to dryness and then methylated in methanol with ethereal diazomethane. Evaporation of the solvents yielded the neutral methyl ester as a gum. (This crystallized on one occasion but was not analysed and it showed $[\alpha]_D^{20°}$ −63° in ethanol and m.p. 123° not sharp.) The ester was acetylated with 4 ml. acetic anhydride and 1 drop of 60% perchloric acid, and after 0·5 hr. the mixture was diluted with water to yield 0·5 g. of crystalline *triacetyl β-o-phenylphenylglucuronide methyl ester*, m.p. 132° and $[\alpha]_D^{20°}$ −85·3° with no mutarotation (*c* = 1 in $CHCl_3$). (Found: C, 61·4; H, 5·5. $C_{25}H_{26}O_{10}$ requires C, 61·7; H, 5·4%.) On treatment with methanolic ammonia for 14 hr. the compound yielded *triacetyl β-o-phenylphenylglucuronidamide* in small yield. This formed colourless plates, m.p. 192–196° and $[\alpha]_D^{25°}$ −68° (*c* = 1 in ethanol). (Found: C, 60·7; H, 5·6; N, 2·8. $C_{24}H_{25}O_9N$ requires C, 61·1; H, 5·4; N, 3·0%.)

p-*Derivatives.* *p*-Phenylphenylglucuronide (m.p. 183° and $[\alpha]_D^{25°}$ −85·3°, 1% in ethanol) was prepared biosynthetically according to Dodgson, Garton, Stubbs & Williams (1948). On methylation with diazomethane it yielded the crystalline *methyl ester*, m.p. 192–193° and $[\alpha]_D^{20°}$ −81·8° (*c* = 1 in ethanol). (Found: C, 61·4; H, 5·4. $C_{19}H_{20}O_7$, 0·5 H_2O requires C, 61·8; H, 5·7%.) Acetylation of this ester (0·75 g.) yielded *triacetyl β-p-phenylphenylglucuronide methyl ester* (0·85 g.) m.p. 170–171° (from aqueous methanol) and $[\alpha]_D^{20°}$ −30·3° (*c* = 1 in $CHCl_3$). (Found: C, 61·4; H, 5·3%.) On deacetylation with methanolic ammonia, *p-phenylphenylglucuronidamide* was obtained as a very insoluble white crystalline powder, m.p. 277° (decomp.). It was too sparingly soluble to permit measurement of its rotation. (Found: C, 62·8; H, 5·6. $C_{18}H_{19}O_6N$ requires C, 62·6; H, 5·5%.) Treatment of this amide with acetic anhydride and a trace of perchloric acid followed by dilution of the acetylation mixture with water yielded *triacetyl p-phenylphenylglucuronidamide* as colourless needles (from ethanol), m.p. 207–210° and $[\alpha]_D^{20°}$ −14° (*c* = 2 in $CHCl_3$). (Found: C, 60·8; H, 5·2. $C_{24}H_{25}O_9N$ requires C, 61·1; H, 5·3%.)

Derivatives of the glucuronides of catechol, resorcinol and quinol (with B. Spencer)

β-o-Methoxyphenylglucuronidamide. This *compound* was prepared by the action of ammonia on a methanolic solution of triacetyl *o*-methoxyphenylglucuronide methyl ester (Garton & Williams, 1948). It crystallized from the reaction mixture as colourless needles, m.p. 216–217° (after recrystallization from 90% ethanol) and $[\alpha]_D^{22°}$ −84° (*c* = 0·2 in methanol). (Found: C, 50·1; H, 5·8. $C_{13}H_{17}O_7N$, 0·5 H_2O requires C, 50·6; H, 5·9%.) Acetylation of this compound (0·1 g.) with pyridine and acetic anhydride gave *triacetyl β-o-methoxyphenylglucuronidamide* (0·102 g.) as colourless needles (from aqueous ethanol), m.p. 144° and $[\alpha]_D^{22°}$ −61·4° (*c* = 0·2 in $CHCl_3$). (Found: C, 53·2; H, 5·2. $C_{19}H_{23}O_{10}N$ requires C, 53·6; H, 5·4%.)

β-m-Hydroxyphenylglucuronidamide. Deacetylation of triacetyl *m*-acetoxyphenylglucuronide methyl ester (1 g.) (Garton & Williams, 1949) with methanolic ammonia yielded *β-m-hydroxyphenylglucuronidamide* (0·512 g.) as colourless needles (from ethanol-ether), m.p. 213° and $[\alpha]_D^{22°}$ −81·6° (*c* = 0·25 in methanol). (Found: C, 49·0; H, 5·5; N, 5·2. $C_{12}H_{15}O_7N$, 0·5 H_2O requires C, 49·2; H, 5·7; N, 4·8%.) On methylation, this compound (0·3 g.) yielded *β-m-methoxyphenylglucuronidamide* (0·305 g.), m.p. 210–211° and $[\alpha]_D^{22°}$ −75·5° (*c* = 0·25 in methanol). (Found: C, 50·9; H, 5·7.) The last compound (0·1 g.) on acetylation yielded *triacetyl β-m-methoxyphenylglucuronidamide* (0·107 mg.) as colourless needles (aqueous ethanol), m.p. 127° and $[\alpha]_D^{22°}$ −12·9° (*c* = 0·3 in ethanol) and −26·3° (*c* = 0·3 in $CHCl_3$). (Found: C, 53·4; H, 5·3%.)

β-p-Hydroxyphenylglucuronidamide. In a similar manner, *β-p-hydroxyphenylglucuronidamide*, m.p. 227–230° and $[\alpha]_D^{22°}$ −85·7° (*c* = 0·25 in methanol) (Found: C, 48·5; H, 5·4; N, 5·0), *β-p-methoxyphenylglucuronidamide*, m.p. 238–240° and $[\alpha]_D^{25°}$ −81·7° (*c* = 0·14 in methanol) (Found: C, 51·4; H, 5·7. $C_{13}H_{17}O_7N$ requires C, 52·0; H, 5·7), and *triacetyl β-p-methoxyphenylglucuronidamide*, m.p. 143–144° and $[\alpha]_D^{24°}$ −28·7° (*c* = 0·25 in $CHCl_3$) (Found: C, 53·5; H, 5·6%) were prepared from triacetyl 4-acetoxyphenylglucuronide methyl ester (Garton & Williams, 1949).

DISCUSSION

The molecular rotations in chloroform of the triacetyl methyl esters of some substituted β-phenyl-D-glucuronides are given in Table 1, from which it is clear that the molecular rotations, $[M]_D$, of the *para*-substituted derivatives (approx. −13000 to

Table 1. *Molecular rotations of the triacetyl methyl esters of substituted β-phenyl-D-glucuronides*

([M]$_D$ measured in 1% solution in $CHCl_3$; temperature 20° in most cases and up to 25° in others. Values for atomic and group radii and for interatomic distance from Wheland (1949) and Remick (1949).)

Substituent group	[M]$_D$/100 *ortho*	*meta*	*para*	Δ[M]$_D$/100 *o–p*	Atomic or group radius (A.)	Interatomic distance (A.)	References to rotations
H	−135	−135	−135	0	0·94	1·08	Parke & Williams (1951)
F	−192	—	−142	− 50	1·39	1·34	This paper
Cl	−288	—	−146	−142	1·89	1·69	This paper
Br	−316	−162	−136	−180	2·11	1·88	This paper
I	−339	—	−138	−201	2·20	2·00	This paper
CH_3	−199	−107	−152	− 47	1·73	—	This paper
C_6H_5	−415	—	−147	−268	—	—	This paper
CN	−309	−161	−183	−126	—	—	Smith (1949)
NO_2	+ 84	−194	−232	+316	1·92	—	Robinson *et al.* (1951)

−15,000), except *p*-NO_2 and *p*-CN, are not very different from that of the unsubstituted triacetyl phenylglucuronide methyl ester (−13,500). The molecular rotations for the *ortho*-substituted derivatives, however, are much more negative than those of the *para*-isomers. This suggests that the *o*-substituents may be involved in steric hindrance with the acetylated sugar ring and so restrict the free rotation of the phenyl ring about the bonds joining it with the glycosidic oxygen. Examination of molecular models shows that steric hindrance might be expected between the *ortho* substituent and the acetoxy group on C-2 of the glucuronic acid; there is apparently little or no hindrance between the *ortho*-substituent and a hydroxyl group on C-2. Figs. 1 and 2 are drawings made from models of *o*-chlorophenylglucuronide and its triacetyl derivative, using the ball and spoke models described by Wooster, McGowan & Moore (1949) and made by Crystal Structures Ltd., Cambridge. The pyranose ring of the glucuronide is shown in that chair conformation which makes all the substituents equatorial, since Reeves (1950) has shown that this is the stable conformation for the hexopyranosides.

Examination of Stuart models of the type described by Settatree, Thomas & Yardley (1950) and made by Catalin Ltd., Waltham Abbey, Essex, confirmed these views on steric hindrance.

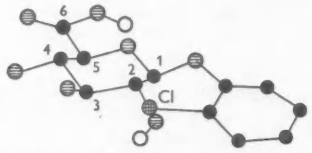

Fig. 1. Drawing made from a ball and spoke model of β-*o*-chlorophenylglucuronide. H atoms on the benzene and carbohydrate rings have been omitted for the sake of clarity. ● = C; ⊜ = O; ⊞ = Cl; ○ = H (see text).

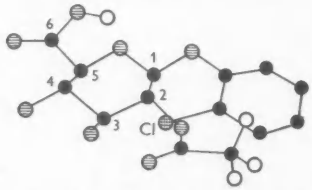

Fig. 2. Drawing of triacetyl β-*o*-chlorophenylglucuronide. Acetyl groups on C-3 and C-4 of the glucuronide have been omitted; other atoms omitted as in Fig. 1.

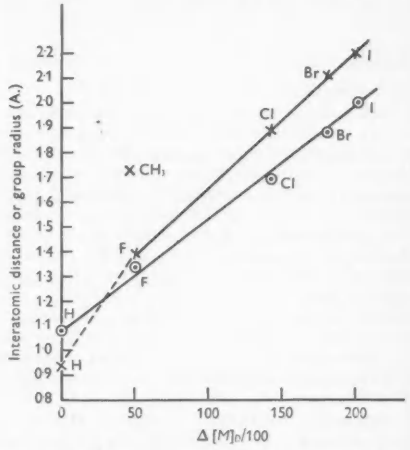

Fig. 3. The relation between the differences in molecular rotation, Δ[M]$_D$/100 between the *ortho*- and *para*-derivatives of the triacetyl methyl esters of halogen substituted β-phenyl-D-glucuronides, and the halogen-carbon interatomic distances (⊙—⊙) or the atomic (or group) radii (×—×).

Some support for the view that steric hindrance is involved has been obtained with the halogen substituted derivatives by plotting the carbon-halogen interatomic distances (taken from Wheland, 1944) against the differences in molecular rotation

Table 2. *Molecular rotations of substituted β-phenyl-D-glucuronidamides in methanol*

Substituent group	$[M]_D/100$ *ortho*	*meta*	*para*	*para*-glucuronide*	References
H	−188	−188	−188	−244	Parke & Williams (1951)
F	−204	—	−221	—	Garton, Robinson & Williams (1949)
Cl	−217	−257	−260	−250	Spencer & Williams (1950)
Br	−225†	−209†	−212†	−238	This paper
CH_3	−201‡	−194‡	−217†	−215	This paper and Bray, Thorpe & White (1950)
OH	—	−240	−252	—	This paper
OCH_3	−251	−233	−244	—	This paper
CN	−236†	−197	−218†	−288	Smith (1949)
NO_2	−200‡	−294	−319	—	Robinson *et al.* (1951)

* The molecular rotation in water of the free *p*-glucuronide is included here for comparison.
† In water.
‡ In ethanol.

($\Delta[M]_D$) between the *ortho*- and *para*-derivatives (see Fig. 3). A straight-line relationship is obtained showing that $\Delta[M]_D$ is proportional to the size of the *o*-substituent. A similar result is obtained when atomic radii (Wheland, 1949) are plotted against $\Delta[M]_D$. In this case we can include the CH_3 group for which a group radius has been quoted. It is to be noted, however, that it does not fall in line with the halogens for its $\Delta[M]_D$ is much less than expected if the relationship given in Fig. 3 is true. This may be because the CH_3 group can be distorted. Table 2 gives the molecular rotations of some non-acetylated phenylglucuronidamides and glucuronides and shows that the molecular rotations of the *o*-, *m*- and *p*-derivatives are roughly the same, suggesting that the steric effect noted above only occurs when the glucuronide is acetylated. The effect, however, shows up again in the triacetyl amides, although here we can only quote three examples (Table 3).

Table 3. *Molecular rotations of some triacetyl β-phenyl-D-glucuronidamides*

(Solvent, $CHCl_3$—except for chloro compounds, which are in ethanol.)

Substituent group	$[M]_D/100$ *ortho*	*meta*	*para*	Reference
H	−130	−130	−130	Parke & Williams (1951)
Cl	−194	− 57	− 46	Spencer & Williams (1950)
OCH_3	261	−112	−122	This paper
C_6H_5	−320	—	− 66	This paper

The diphenyl derivatives

Much of our knowledge of the phenomenon of restricted rotation has been obtained from the study of substituted diphenyl derivatives (for reviews see Wheland, 1949; Barnett, 1950). We therefore prepared the triacetyl methyl esters of the glucuronides of *o*- and *p*-hydroxydiphenyl and found that whereas the *p*-derivative shows a normal molecular rotation (−14,700) the *o*-derivative possesses an abnormally high negative rotation (−41,500) which is nearly three times the $[M]_D$ of the *para*-isomer. Here we could have not only restricted rotation of the bonds *a* and *b* between the carbohydrate ring and its attached phenyl group but also restricted rotation between the two phenyl rings at bond *c*. These possibilities can be visualized from Figs. 4 and 5. However, an examination of the spectra of

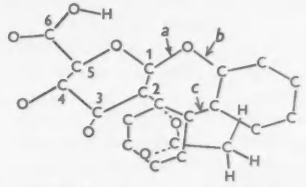

Fig. 4. Drawing of triacetyl β-*o*-phenylphenylglucuronic acid. Atoms and groups omitted as in Fig. 2.

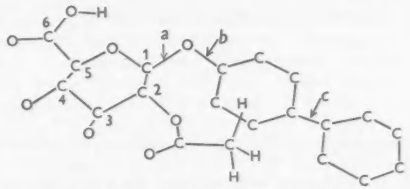

Fig. 5. Drawing of triacetyl β-*p*-phenylphenylglucuronic acid. Atoms and groups omitted as in Fig. 2.

the hydroxydiphenyls and the triacetyl methyl esters of their glucuronides (see Table 4) suggests that in the diphenyl system of both acetylated glucuronides the two benzene rings are coplanar. The molecular extinctions ($\epsilon_{max.}$) of the main bands of these glucuronides (*o*-, 12,600 and *p*-, 20,200) are of the same order as that of diphenyl itself (20,000), the high extinction being due to pronounced conjugation between the two aromatic rings (Braude, 1945) which is only possible if they are coplanar. If

Table 4. *Absorption spectra of the hydroxydiphenyls and their glucuronides*

(Solvent, ethanol—except for benzene and diphenyl which were measured in hexane.)

Compound	$\lambda_{max.}$ (mμ.)	$\epsilon_{max.}$	Reference
Benzene	256	250	Braude (1945)
Diphenylmethane	261	502	Magat & Maier (1943)
Diphenyl	246	20,000	Braude (1945)
p-Hydroxydiphenyl	260	17,600	This paper
Triacetyl methyl ester of β-*p*-glucuronosidodiphenyl	252–8	20,200	This paper
o-Hydroxydiphenyl	246 (290)*	10,000 (4,700)	This paper
Triacetyl methyl ester of β-*o*-glucuronosidodiphenyl	245 (278)*	12,600 (2,600)	This paper

* Secondary band.

they were not coplanar, resonance between the two rings would be prevented and the molecular extinctions might be expected to be lower, perhaps about 500 (as in diphenylmethane) which is the sum of the extinctions of the 256 mμ. band of two separate benzene rings. Wheland (1944) quotes among other examples dimesityl, whose absorption spectrum is almost identical (per aromatic ring) with that of mesitylene but very different from that of diphenyl itself (Pickett, Walter & France, 1936; O'Shaughnessy & Rodebush, 1940). In dimesityl planar arrangement of the two rings is made impossible by the steric interactions of methyl groups. The abnormal optical rotation of triacetyl β-*o*-phenylphenylglucuronide methyl ester would therefore appear to be due to hindered rotation about the glycosidic linkage. The glucuronide showed no mutarotation.

SUMMARY

1. A number of phenols have been fed to rabbits and the β-glucuronides have been isolated from the urine. The glucuronides have been converted to amides and triacetyl methyl esters. The phenols used were *o*- and *p*-fluoro-, *o*- and *p*-chloro-, *o*-, *m*- and *p*-bromo- and *o*- and *p*-iodophenols; *o*-, *m*- and *p*-cresols; *o*- and *p*-hydroxydiphenyls. Seventeen new triacetyl methyl esters are described.

2. The molecular rotations of these compounds have been determined and the triacetyl methyl esters of most of the *o*-substituted phenylglucuronides show higher negative rotations than the corresponding *m*- and *p*-isomers. This effect is not shown by the non-acetylated glucuronidamides.

3. In the case of the triacetyl methyl esters of the *o*-halogenophenylglucuronides, it appears that the molecular rotation is directly related to the atomic radius of the substituent or to the length of the carbon-halogen link. The abnormality may be due to restricted rotation about the bond joining the phenyl group to the glycosidic oxygen atom.

4. The abnormally high negative rotation of the triacetyl methyl ester of β-*o*-phenylphenylglucuronide as compared with its *p*-isomer suggests that the phenomenon of restricted rotation may be involved here also. Spectroscopic data suggest that there is no restriction of rotation between the two phenyl rings of this compound.

We are grateful to Dr W. Klyne for valuable discussions about atomic models and to Mr R. V. Brooks for photographs on which Figs. 1 and 2 are based.

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Ethanolamine *O*-Phosphoric Acid in Rat Brain

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The presence of ethanolamine phosphoric acid in brain tissue was first suggested by the work of Stone (1943), who found that a small fraction of the acid-soluble phosphorus compounds in dog brain possessed properties similar to those of this substance. Using chromatographic methods, Awapara, Landua & Fuerst (1950) have reported the presence of the compound in rat brain. The biochemical interest of ethanolamine phosphoric acid in tissues lies in its possible role as an intermediary in the metabolism of the phospholipins. From the results of experiments with ethanolamine phosphoric acid labelled with radioactive phosphorus, ^{32}P, Chargaff & Keston (1940) concluded that the compound is not an intermediary in the synthesis of phospholipins, and suggested that it might arise in the tissues by the breakdown of phosphatidyl ethanolamine. In this connexion, Tyrrell (1950) has recently described a 'cephalinase' which is present in nervous tissue.

In the present investigation the presence of free ethanolamine phosphoric acid in rat brain has been confirmed, and the uptake of radioactive phosphorus into this compound has been measured. The results suggest that the major portion of the ethanolamine phosphoric acid present in rat brain is not produced by the breakdown of phosphatidyl ethanolamine. Experiments have also shown that synthesis of ethanolamine phosphoric acid can take place in isolated brain tissue by a process which appears to be independent of the breakdown of phosphatidyl ethanolamine.

EXPERIMENTAL

Young rats (30–40 g.) were used throughout the investigation. For experiments *in vivo* these were injected intraperitoneally with a solution of KH_2PO_4 containing 40–280 μc.^{32}P. After the experimental period they were stunned and immediately immersed in liquid oxygen. The frozen whole brain was removed, crushed, weighed on a torsion balance and transferred to a centrifuge tube containing 5 ml. of water at 100°. To the hot aqueous suspension was added the requisite amount of tungstic acid for protein precipitation, as recommended by Schurr, Thompson, Henderson & Elvehjem (1950) (i.e. 0·6 ml./g. of brain, of a solution prepared by mixing 5 vol. of 10% sodium tungstate and 7 vol. of 0·6 N-H_2SO_4). The suspension was kept at 100° for 2 min. and stood for at least 50 min. at room temperature before centrifuging.

Chromatographic isolation of ethanolamine phosphoric acid phosphorus. The suspension of brain tissue was centrifuged, and approximately 4 ml. of the supernatant desalted in an apparatus similar to that used by Consden, Gordon & Martin (1947). Distilled water was used in the anode compartment and was changed several times during the course of the desalting. A large portion of the radioactivity of the extract was removed by the process. The percentage of the radioactivity remaining after the desalting increased with the length of time allowed for ^{32}P exchange in the animal.

Samples of the desalted extract equivalent to 0·05 g. brain were subjected to two-dimensional chromatography. Whatman no. 4 paper was used because it had a lower P content than Whatman no. 1 paper. It was irrigated first with water-saturated phenol and then with water-saturated 'collidine/lutidine' (Dent, 1948), but ammonia and diethylamine were not added to the chromatography cabinet. The chromatograms were dried below 70° after each irrigation and then sprayed with 0·1% ninhydrin in water-saturated butanol. After development overnight at room temperature the ethanolamine phosphoric acid spot (*X* in Fig. 1) was cut out, together with an equivalent area around the spot which served as a control. (This equivalent area was measured by weighing on a torsion balance.) The spots and associated paper from two chromatograms were combined and oxidized by heating with 2 ml. of 60% (w/w) perchloric acid; the reaction was completed by the addition of 0·2 ml. 30% (w/v) H_2O_2 and 0·05 ml. HNO_3 when necessary. (Precautions were taken to prevent personal injury in the event of an explosion.) The residual solution was evaporated to approximately 0·6 ml.; made up to 10 ml. with distilled water and determinations made of the radioactivity and phosphorus content. The control area of paper was assayed in a similar manner.

Ether-soluble phospholipin phosphorus. The precipitate obtained on centrifuging the brain suspension was treated with 30 ml. of 5% trichloroacetic acid solution and, after re-centrifuging, the residue was stirred with 30 ml. water. After standing overnight at 0° the residue was rewashed with 30 ml. water, then twice with 15 ml. volumes of acetone at 0°. The residue was slowly dried at 37° and the phospholipins extracted by refluxing for 30 min. with 25 ml. of a 50% (v/v) $CHCl_3$-ethanol mixture. The solution of phospholipins was evaporated to dryness under reduced pressure and the residue treated with 15 ml. of cold diethyl ether. After centrifuging, the ether was removed from the supernatant by evaporation. The ether-soluble phospholipin residue was oxidized with perchloric acid and H_2O_2, and determinations made of the radioactivity and P content.

The supernatant from a tungstic acid precipitation of brain suspension contained a negligible amount of ether-soluble phospholipins, indicating that the precipitation was complete.

Inorganic + acid-labile phosphorus. A sample (1 ml.) of the supernatant from the tungstic acid precipitation was hydrolysed with N-HCl for 10 min. at 100°. After cooling, 1 ml. of 0·8 M-$CaCl_2$ was added and the pH adjusted to 8·5 with NaOH solution. The suspension was kept at 0° for

15 min., centrifuged and the residue suspended in 7 ml. of distilled water. Perchloric acid (0·6 ml. of 60%, w/w) was added and then 2·5 ml. of 5% (w/v) ammonium molybdate solution. The phosphomolybdic acid was extracted into 10 ml. of *iso*butanol. The extract was then washed with an equal volume of N-H_2SO_4 and made up to 10 ml. with *iso*butanol. The ethanolic solution was assayed for radioactivity and the phosphomolybdic acid then reduced with $SnCl_2$ to measure the phosphate colorimetrically.

In some experiments the hydrolysis with N-HCl was omitted, but the high value of the 'inorganic P' figure showed that very considerable hydrolysis of the labile phosphoric esters had occurred during the experimental procedure. It is known that there is an extremely rapid turnover of the P in these labile phosphoric esters of the brain (Linberg & Ernster, 1950), and there would probably be little difference between the specific activities determined with and without preliminary hydrolysis with N-HCl.

Determination of the radioactivity. The procedure and corrections used have been described in a previous paper (Dawson & Richter, 1950). As the inorganic and acid-labile P were assayed in *iso*butanol, a small correction was applied to allow for the different absorption of β-radiation by this solvent. All counting rate measurements were referred to a solution containing 5% (w/w) perchloric acid. For convenience, the specific activity used was calculated as the counting rate/mg. P.

Phosphorus determinations. The method of Berenblum & Chain (1938) was used, except in experiments *in vitro* when the inorganic and ether-soluble phospholipin P was estimated by the procedure of Fiske & Subbarow (1925). Both methods have the advantage that the colour development is not influenced by large variations in the concentration of perchloric acid used.

Experiments in vitro. Experiments demonstrating ethanolamine phosphoric acid synthesis by isolated brain tissue were carried out in Warburg vessels. The rats were killed by decapitation, the whole brains finely minced with a razor and suspended in Krebs bicarbonate-Ringer solution (200 mg. wet weight brain tissue/ml.). After incubation in the presence of labelled KH_2PO_4 the contents of each Warburg vessel were treated with 0·22 ml. of tungstic acid solution and heated at 100° for 5 min.

The suspension was centrifuged and the inorganic and labile P estimated in a 0·4 ml. sample of the supernatant. The rest of the supernatant was treated with enough solid $Ca(OH)_2$ to make a saturated solution, and the precipitated calcium phosphate removed by centrifuging. Excess $Ca(OH)_2$ was removed as $CaCO_3$ by saturating with CO_2 and boiling. The solution was desalted and subjected to chromatography to isolate the ethanolamine phosphoric acid. Ether-soluble phospholipins were isolated by a procedure similar to that described previously, except that the tungstic acid precipitate was given additional washes with 5% trichloroacetic acid solution and water.

Synthesis of ethanolamine phosphoric acid. Ethanolamine phosphoric acid was prepared by the method of Outhouse (1937), m.p. 241° (uncorr.).

RESULTS

Chromatographic separation of ethanolamine phosphoric acid. Two-dimensional chromatograms of trichloroacetic acid and tungstic acid extracts of whole rat brain frozen *in situ* with liquid oxygen, showed a relatively large spot (*X*) under the glutamic acid spot (Fig. 1). The intensity of this spot was reduced in chromatograms of extracts prepared from autolysed brain. With extracts from human brain, several days post-mortem, only a very faint spot was observed. The spot was not seen on chromatograms of acid hydrolysates of rat brain proteins.

The ninhydrin-reacting material which formed spot *X* was judged to contain ethanolamine phosphoric acid from the following observations:

(1) The material showed identical chromatographic behaviour to that of synthetic ethanolamine phosphoric acid.

(2) It was highly stable to acid hydrolysis. After 24 hr. hydrolysis of brain extracts at 100° in 5·5N-hydrochloric acid a considerable portion of spot *X* survived. On prolonging the hydrolysis for 72 hr. a faint spot was still visible, but a new spot appeared on the chromatogram. This new spot occupied the same position as free ethanolamine and was still present when the chromatogram was treated initially with basic copper carbonate to eliminate α-amino-acids (Crumpler & Dent, 1949).

(3) The spot was still present after the brain extract had been hydrolysed for 67 hr. at 100° in N-sodium hydroxide. All the spots on this chromatogram were of reduced intensity due possibly to the removal of the amino-acids by the silica gel produced on neutralization of the sodium silicate formed by the action of the alkali on the glass container. Plimmer & Burch (1937) have reported that synthetic ethanolamine phosphoric acid is very stable to hydrolysis with N-sodium hydroxide.

(4) On preliminary treatment of the chromatogram with basic copper carbonate, according to the procedure of Crumpler & Dent (1949), the spot was still visible, together with those of taurine and γ-aminobutyric acid.

(5) Analysis of the spot cut from the chromatogram showed that it contained an amount of phosphorus significantly higher than that in an equal area of paper surrounding the spot.

(6) In further experiments measurements were made of the intensity of the ninhydrin colour produced by extracting the spot with water according to the method of Roberts & Frankel (1950). The aqueous extract and the filter-paper pulp were then combined and analysed for phosphorus. It was found that the ratio between the intensity of the ninhydrin colour and the phosphorus content was identical, within the limits of experimental error, with that obtained when synthetic ethanolamine phosphoric acid was subjected to the same procedure.

Identification of the unknown spot by an isotopic procedure. To confirm the presence of ethanolamine

phosphoric acid in rat brain, a rat under ether anaesthesia was injected intracisternally with 100 μc. of ^{32}P. After 4 hr. the animal was decapitated, the head immediately immersed in liquid oxygen, and a tungstic acid extract of the brain prepared. After desalting, the solution was subjected to two-dimensional paper chromatography and the unknown spot *X* located with acid. This evidence confirmed that the unknown spot *X* on chromatograms of rat-brain extracts consisted of ethanolamine phosphoric acid.

The uptake of ^{32}P into the ethanolamine phosphoric acid of the brain in vivo. In experiments in which the uptake of ^{32}P into the ethanolamine phosphoric acid of the brain *in vivo* was determined, it was found necessary to inject the rats with phosphate of a high

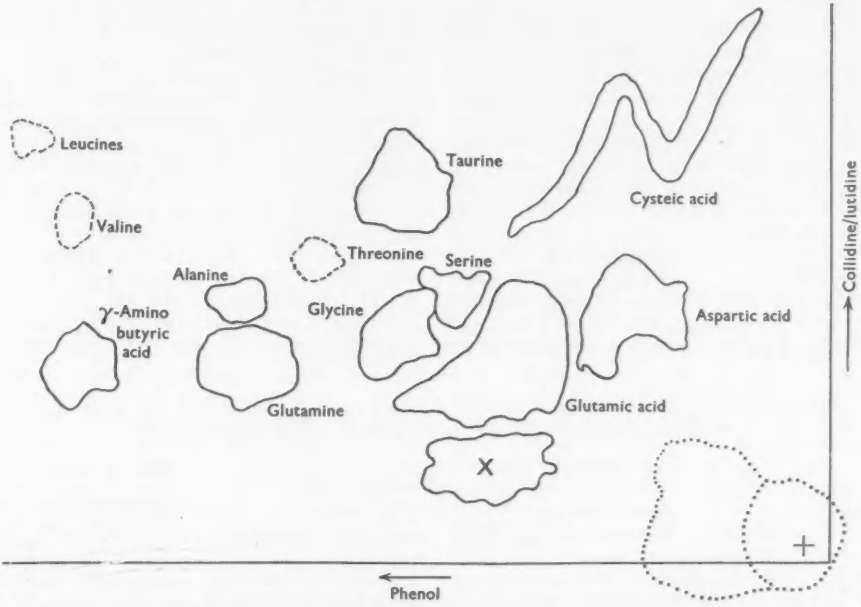

Fig. 1. Typical pattern of ninhydrin-reacting substances produced by the two-dimensional chromatography of rat-brain extract (equivalent to 0·04 g. wet tissue) which had previously been oxidized with H_2O_2. Interrupted lines indicate faint spots. Dotted line indicates the limit of the spread of inorganic phosphate and acid-labile phosphate when the extract had not been desalted to remove these substances.

ultraviolet light (Woiwod, 1949; Fowden, 1951). A solution of a small quantity of the unknown substance (equivalent to 0·1 g. wet brain tissue) was obtained by elution of the chromatographic spots with boiling water. After evaporation to 2 ml., 150 mg. synthetic ethanolamine phosphoric acid was dissolved in the solution, and then crystallized out by the addition of ethanol to 80 %. The specific activity of the product was determined. This process of recrystallization from 80 % ethanol was repeated a further five times during which the specific activity remained absolutely constant. The final product was then converted into the flavianic acid derivative (Outhouse, 1936) and crystallized from aqueous *n*-butanol, m.p. 225°. The specific activity of the phosphorus in this compound was identical with that in the original ethanolamine phosphoric activity in order to ensure a measurable counting rate from the ethanolamine phosphoric acid spot. As the blood-brain barrier is not very permeable to injected phosphate it is unlikely that the phosphorus metabolism of the brain would be appreciably affected by the radiation.

Experience showed that tungstic acid extracts of whole brain tended to give better defined ethanolamine phosphoric acid spots than trichloroacetic acid extracts. The area of paper around the ethanolamine phosphoric acid spot possessed no detectable radioactivity and its phosphorus content approximated closely to that of the paper blank.

Chromatograms of brain extracts, which had not been desalted, were examined for inorganic phosphate and phosphoric esters by the method of Hanes & Isherwood (1949). Part of these substances

remained at the point of application of the extract and part trailed in the phenol run to a position half way between the ethanolamine phosphoric acid spot and the origin (Fig. 1). Movement in the collidine/lutidine run was negligible. No inorganic phosphate was detected by this method at the position of the ethanolamine phosphoric acid spot, presumably because of its high stability to acid hydrolysis. No inorganic phosphate or phosphate esters could be detected on the chromatograms of desalted brain extracts. When chromatograms of a mixture of synthetic ethanolamine phosphoric acid and radioactive inorganic phosphate were run, the ethanolamine phosphoric acid spot showed no radioactivity.

The results presented in Table 1 show that, after ^{32}P had been allowed to 'exchange' within the animal for 3 hr., the ethanolamine phosphoric acid in the brain had a specific activity approximately 50–70% of that of the inorganic + acid-labile P fraction and fifteen to nineteen times greater than the specific activity of the corresponding ether-soluble phospholipin fraction. In two experiments in which shorter times (45 and 60 min.) were allowed for phosphorus 'exchange', the ratios of the specific activities of the ethanolamine phosphoric acid and the ether-soluble phospholipins were even greater (Table 1).

The synthesis of ethanolamine phosphoric acid by minced rat-brain tissue. In preliminary experiments designed to show the synthesis of ethanolamine phosphoric acid *in vitro*, minced rat-brain tissue was suspended in glucose-phosphate-Ringer solution and incubated under conditions which ensured a high rate of respiration. The solution obtained after protein removal with tungstic acid was desalted and subjected to two-dimensional chromatography. It was found that the intensity of the ethanolamine phosphoric acid spot did not increase above that of a control when 0·03 M-ethanolamine hydrochloride was added to the brain suspension prior to the incubation. Similar results were obtained when the experiments were carried out in the presence of 0·016 M-sodium fluoride. However, the possibility existed that the detection of an increased ethanolamine phosphoric acid production by this method would be prevented by the breakdown of the compound by phosphatase activity. Consequently, experiments were made in which the minced brain tissue was suspended in glucose-bicarbonate-Ringer

Table 1. *Specific activities of the ethanolamine phosphoric acid, ether-soluble phospholipins and inorganic + acid-labile phosphate in rat brain after the injection of* ^{32}P

Rat no.	Wt. of rat (g.)	Duration of experiment (min.)	Dose ^{32}P (μc./g.)	Specific activity of: Ethanolamine phosphoric acid P (counts/min./mg. P)	Specific activity of: Ether-soluble phospholipin P (counts/min./mg. P)	Specific activity of: Inorganic + acid-labile P (counts/min./mg. P)	Ratio of specific activities: Ethanolamine phosphoric acid P/ether-soluble phospholipin P	Ratio of specific activities: Ethanolamine phosphoric acid P/inorganic + acid-labile P
1	35·0	180	1·7	11,820	—	15,850	—	0·74
2	37·0	45	1·2	1,785	30	8,130	59	0·22
3	30·6	100	1·3	1,730	109	5,500	16	0·31
4	38·6	185	1·1	2,460	165	4,850	15	0·51
5	37·8	60	7·4	8,650	335	33,400	26	0·26
6	34·3	120	7·3	12,400	732	37,000	17	0·33
7	37·5	180	7·5	22,200	1,172	37,000	19	0·60

Table 2. *Synthesis of ethanolamine phosphoric acid by minced rat brain*

(Each Warburg vessel contained 340 mg. minced rat brain; 3·35 ml. Krebs bicarbonate-Ringer solution; 4 μc. ^{32}P as KH_2PO_4 (0·03 mg. P). Glucose concentration 0·018 M. Temp. 37·5°. Gas phase 95% O_2, 5% CO_2.)

Time of incubation (min.)	Additional substrate	Specific activity of: Ethanolamine phosphoric acid P (counts/min./mg. P)	Specific activity of: Ether-soluble phospholipin P (counts/min./mg. P)	Specific activity of: Inorganic P + acid-labile P (counts/min./mg. P)	Ratio of specific activities of ethanolamine phosphoric acid P/ether-soluble phospholipin P
0	—	0	350	191×10^4	0
60	—	>7,200	1,880	186×10^4	>3·8
180	—	10,700	2,370	186×10^4	4·5
180	0·0035 M-Ethanolamine HCl	29,400	2,270	176×10^4	13·0

solution and incubated aerobically with radioactive potassium dihydrogen phosphate. The ethanolamine phosphoric acid isolated in these experiments was found to be radioactive (Table 2), demonstrating that synthesis had occurred, and its specific activity was higher than that of the ether-soluble phospholipins, isolated from the same sample of tissue. Moreover, when 0·0035 M-ethanolamine hydrochloride was present during the incubation period, the synthesis of ethanolamine phosphoric acid was accelerated so that after 2 hr. its specific activity was thirteen times greater than that of the corresponding ether-soluble phospholipins.

The value for the specific activity of the ether-soluble phospholipin phosphorus at zero incubation time given in Table 2, points to the extreme difficulty of freeing phospholipins from contaminating inorganic phosphate. Fries, Schachner & Chaikoff (1942) found the same difficulty in their experiments on the uptake of ^{32}P into the phospholipins of surviving brain tissue.

Concentration of ethanolamine phosphoric acid in whole rat brain. The recovery of phosphorus from spot *X* corresponded to a mean value (eight brains) of 9 mg. ethanolamine phosphoric acid/100 g. whole brain, though individual values showed a wide variation from 4 to 13 mg./100 g. brain. Awapara *et al.* (1950) record a minimal content of 11 mg./100 g. for adult rat brain obtained by estimation of the spot on chromatograms with ninhydrin.

When 10 μg. ethanolamine phosphoric acid standards were run on a two-dimensional chromatogram, the recovery of phosphorus from the spot was only about 60 %. The recovery was little improved by drying off the solvents from the chromatogram at room temperature. Fowden (1951) has recently shown that when the amino-acids are estimated colorimetrically with ninhydrin the recoveries are greatly improved by low temperature drying of the chromatograms. Further experiments showed that although ethanolamine phosphoric acid hardly moved in the collidine/lutidine run, some loss of phosphorus occurred; a washing-out process may account for this observation. Visible tailing of the ethanolamine phosphoric acid spot occurred in the phenol run. These results indicate that estimation of the phosphorus content of the ethanolamine phosphoric acid spot on a two-dimensional chromatogram irrigated first with phenol and then collidine/lutidine is not a satisfactory method for the quantitative estimation of the substance in tissues and the results only indicate minimal values.

DISCUSSION

There is now good evidence that the ninhydrin-reacting substance occurring below the glutamic acid spot on two-dimensional chromatograms of brain extracts consists of ethanolamine phosphoric acid. As the spot is present on chromatograms of extracts prepared after the freezing of the brain *in situ* with liquid oxygen, the substance is not likely to have been formed by post-mortem enzymic activity. It has been suggested by Chargaff & Keston (1940) that ethanolamine phosphoric acid arises in tissues by the enzymic breakdown of phosphatidyl ethanolamine. The present experiments showed that when rats were injected with potassium dihydrogen phosphate containing radioactive phosphorus and killed after a few hours, the specific activity of the brain ethanolamine phosphoric acid phosphorus was many times greater than the specific activity of the corresponding ether-soluble phospholipin phosphorus. This strongly suggests that the whole or a large portion of the ethanolamine phosphoric acid in the brain does not arise by the catabolism of brain phosphatidyl ethanolamine. It is interesting in this connexion that Sloane Stanley (1951) obtained no evidence of the release of nitrogenous compounds in his studies on the breakdown of kephalin by brain homogenates.

The ether-soluble phospholipin fraction isolated in the present investigation was undoubtedly complex and contained lecithin as well as phospholipins containing serine, ethanolamine and inositol (Folch, 1942). The results of Artom (1945) indicate that the ether-soluble phospholipins isolated from rat brain contain about 40 % of phosphatidyl ethanolamine. Therefore, in the present experiments, even if the entire ^{32}P turnover of this ether-soluble phospholipin fraction was located in the phosphatidyl ethanolamine portion, the resulting specific activity of this compound after 3 hr. exchange would still be six times less than that of the corresponding ethanolamine phosphoric acid. It should not be assumed that the turnover of the phosphatidyl ethanolamine itself is homogeneous. The phospholipins probably exist in the cell in different chemical forms (α and β; lipoproteins) each with its own turnover rate. Furthermore, the turnover rate of the compound in different anatomical and cytological regions may not be constant. However, to account for the results obtained, ethanolamine phosphoric acid could only be produced by phosphatidyl ethanolamine breakdown if it were selectively formed from a portion of the phosphatidyl ethanolamine with a turnover rate very much higher than that of the rest of the fraction.

The question arises whether ethanolamine phosphoric acid is carried to the brain in the blood or synthesized, wholly or partly, in the brain. Radioactive ethanolamine phosphoric acid was isolated from a minced brain suspension respiring in the presence of labelled potassium dihydrogen phosphate and as the specific activity of the substance was

many times higher than that of the corresponding ether-soluble phospholipin fraction, it would seem that it had not arisen by phosphatidyl ethanolamine breakdown. If the radioactive ethanolamine phosphoric acid was produced by a simple phosphorylation, it would appear that the rate of synthesis *in vitro* must be extremely slow. This assumes that the labile phosphate groups of adenosinetriphosphate are as rapidly exchanged *in vitro* as they have been shown to do in the intact brain (Linberg & Ernster, 1950). However, the possibility that ethanolamine phosphoric acid arises from the breakdown of other phosphorus-containing compounds, such as acetal phospholipin (Thannhauser, Boncoddo & Schmidt, 1951), cannot be excluded.

The role of ethanolamine phosphoric acid in brain metabolism is not yet clear. As the formation of the compound in brain tissue appears to be independent of phosphatidyl ethanolamine breakdown and as it has a relatively high phosphorus turnover in the intact brain, the acid cannot be excluded as a precursor of phosphatidyl ethanolamine on the basis of the present results. From the work of Chargaff & Keston (1940) it would appear that ethanolamine phosphoric acid is not an intermediary of phosphatidyl ethanolamine synthesis in the liver or intestinal tract. Their results, however, are not completely conclusive, as our new knowledge of the solubility of the phospholipins (Folch, 1942) creates uncertainty as to the composition of the 'kephalin' fraction isolated by these workers.

SUMMARY

1. Ethanolamine phosphoric acid has been identified in extracts of rat brain frozen *in situ* with liquid oxygen.

2. The recovery of phosphorus from a sample of synthetic ethanolamine phosphoric acid run on a two-dimensional chromatogram irrigated with phenol and collidine/lutidine was not quantitative. Therefore the recoveries from tissue extracts by this chromatographic procedure represent minimal values only.

3. After 3 hr., the specific activity of the ethanolamine phosphoric acid of the brain in the intact rat injected with ^{32}P was approximately 50–70% of the specific activity of the inorganic + acid-labile phosphate fraction and many times greater than the specific activity of the ether-soluble phospholipin fraction.

4. The results suggest that the major portion of the acid had not originated by the breakdown of the phosphatidyl ethanolamine in the brain.

5. The synthesis of ethanolamine phosphoric acid by isolated rat-brain tissue has been demonstrated.

The authors thank Dr D. Richter for his continued interest during the investigation and Mr D. W. Davies for his assistance in chromatographic manipulation. They thank the University of London and the Rockefeller Foundation for apparatus grants. One of us (G.B.A.) is indebted to the Medical Research Council for a research grant.

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The Preparation and Some Properties of Purified Micrococcin

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The formation of the antibiotic micrococcin by a species of *Micrococcus*, since given the number 7218 in the National Collection of Type Cultures, and its extraction from active broth, were described by Su (1948). The properties of his purest preparation, a colourless, amorphous powder, now believed to have been about 85% pure, were also given. The material is almost insoluble in water, but large doses can be given intravenously in finely divided form, the particles being rapidly taken up by the cells of the reticulo-endothelial system and persisting in the body for weeks or months. It can also be solubilized by certain detergents, and the results of animal experiments with particulate and solubilized micrococcin are described elsewhere (Markham, Heatley, Sanders & Florey, 1951; Markham, Wells, Heatley & Florey, 1951; Sanders, Florey & Wells, 1951; Heatley, Gowans & Florey, 1951).

Experience gained during the working up of some of the large batches of material needed for the animal experiments is described below, together with some chemical and physical properties of micrococcin.

EXPERIMENTAL

Methods

Source of crude culture fluid

Some batches of about 110 l. each were set up at Oxford in Lemco broth, as described by Su (1948), in aerated 5–10 l. bottles. In December 1948 two batches of 81 and 84 gal. were prepared in the Clevedon laboratories of the Distillers Co. Ltd.; the crude broth was acidified and after settling, the slurry was sent to Oxford for working up. Further batches were prepared at Clevedon under the control of the Medical Research Council. Later, the whole of the extraction, up to the preparation of a fairly pure amorphous solid, was done at Clevedon. During this work a cheaper medium giving a much enhanced yield of micrococcin was developed, and some observations were made on the biochemistry of the fermentation (Kelly, Miller & Hale, 1951).

Assay

Methods for the assay of micrococcin are reported elsewhere (Heatley, Kelly & Smith, 1951).

Extraction and isolation

Of the three processes described by Su (1948), the precipitation process was the most convenient and was used in the present work, but the following practical points may be worth mention.

General procedure. The crude culture fluid was adjusted to pH 2 and, after settling, the clear supernatant was discarded and the slurry centrifuged. The solid was extracted with ethanol two or three times and the extracts concentrated by vacuum distillation until all ethanol had been driven off; if necessary, water was added as the distillation neared completion. At this stage, depending on the nature and amount of anti-foam agent used in the fermentation, an extraction with ether was sometimes done, the ether being discarded. The watery suspension of pasty solid was rinsed from the flask with $CHCl_3$ into a separating funnel and well shaken with 5–6 successive portions of $CHCl_3$. The $CHCl_3$ extracts were passed through a column of Brockmann type alumina, starting with the last $CHCl_3$ extract. (Alternatively the weakest extracts were kept and used for the first extractions in the next batch.) The column was well washed with fresh $CHCl_3$, the purple fluorescent band was cut out, dried in air, and eluted with ethanol. The band containing the micrococcin was sharpened but not appreciably developed by $CHCl_3$, and each ml. of alumina in this band adsorbed 5–15 mg. of micrococcin. (The fact that the micrococcin was not washed through the column by large volumes of $CHCl_3$ suggested the possibility of an alumina trap as part of a continuous cyclical extraction procedure, but this was not followed up.)

Occasionally the fluorescent band was more diffuse and passed down the column more or less readily. This was traced to the presence of a small amount of ethanol in the $CHCl_3$. Care was therefore taken to remove all ethanol from the ethanolic extracts and from the $CHCl_3$.

Technique of ethanolic extraction. It was difficult to disperse the clay-like centrifuged acid precipitate of the crude culture fluid evenly in the ethanol except on a very small scale, and the extraction was correspondingly incomplete or tedious. A satisfactory solution, which might be of general use, was to allow the original acid precipitate to settle by gravity, then gradually to mix the uncentrifuged creamy slurry with a large volume of ethanol. The extraction was efficient, but a larger volume of extract had to be dealt with, and as the percentage of water was higher it is possible that more or different impurities were extracted.

The most convenient method was the following. The delivery tube of a centrifugal pump was connected to a nozzle of about 4 mm. internal diameter arranged within a large funnel as shown in Fig. 1 A. The stem of the funnel was connected to the intake of the pump, and enough ethanol was added to half fill the funnel and automatically prime the pump. The acid-precipitated solid, whether packed in the bowl of a continuous centrifuge of the Sharples type, or in separate centrifuge bottles, was dislodged by the stream of ethanol from the jet and fell into the funnel, from which it was re-circulated through the pump. After all the precipitate had been dislodged, the suspension was circulated for some minutes, at the end of which time a smooth finely divided suspension containing the minimum amount of water was obtained. After centrifuging, the sediment was

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readily re-suspended in fresh ethanol (for a second extraction) by shaking by hand. For handling larger amounts of precipitate the modification shown in Fig. 1 B was used.

Elution from alumina. The air-dried purple fluorescent zone of the alumina column was poured into another column and percolated with ethanol. The first few ml. of percolate were very concentrated—probably saturated, or nearly so—but became less so as elution proceeded. Although each drop of eluate was always perfectly clear a pad of precipitated amorphous material often formed in the first few ml. of eluate, although if this were collected in two

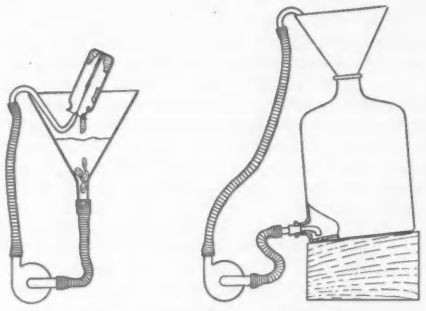

Fig. 1. Methods for suspending initial precipitate in ethanol. For explanation see text.

or three fractions each remained perfectly clear. Micrococcin is more soluble in 90% than in 100% ethanol (see below). When the almost water-free ethanol is passed through the column it probably removes some water from the air-dried alumina and thus acquires an increased solvent power for micrococcin. The water content of the successive eluates falls and the combined total eluate becomes supersaturated with micrococcin, which is precipitated.

Crystallization of micrococcin

Prolonged attempts to crystallize micrococcin were unsuccessful—Su (1948) reported the same—but very small crystals appeared spontaneously in an ethanolic concentrate which had been standing at room temperature for 2 or 3 weeks. In spite of this piece of good fortune it still proved impossible to obtain crystals from some concentrates of high activity and it was uncertain whether there were one or more active substances. Chromatography and an 8-funnel counter-current distribution by the method of Craig, Golumbic, Mighton & Titus (1945) in a mixture of water, $CHCl_3$, carbon tetrachloride, glacial acetic acid and ethanol gave no evidence for the presence of more than one active substance, and it was known that recrystallized material could come out of solution in the form of a gel. In some cases seeding of such a gel with crystals would lead to the gradual and complete conversion of the whole gel to crystalline form, either at room temperature or at 4°. On the other hand, certain gels would be only partly converted to crystals when seeded, no matter at what temperature or for how long they were left.

Another observation suggesting the occurrence of more than one active substance was the apparent variation in solubility of the material from different batches. Thus, it had been known that solutions containing 10 mg. of micrococcin per ml. were supersaturated, for seeding crystals increased in size; in certain other solutions, however, containing double the concentration of substance in terms of dry weight, activity, and fluorescence, similar seeding crystals not only failed to increase in size but actually disappeared. This at first appeared to be almost overwhelming evidence for the occurrence of more than one active substance, but was later explained by the presence of a little more water in the ethanol in the second experiment than in the first. (This could easily happen through the use of different batches of recovered ethanol, as was the practice at the time.)

The difficulty of crystallizing micrococcin seems to be due first to the rather exacting conditions required, and secondly to the presence, at any rate in some preparations, of an impurity which inhibits crystallization. Evidence for such an impurity is the fact that a gel which refuses to crystallize, if filtered as completely as possible from its mother liquor, then redissolved in fresh ethanol, will furnish at any rate a partial crop of crystals. Also, recrystallization of material which has already been crystallized never presents difficulty.

With more experience it has proved possible to obtain crystals regularly, the procedure being as follows:

The crude dry solid is dissolved in sufficient warm azeotropic ethanol to give a solution containing not more than about 20 mg./ml. This is filtered, then placed at 37°. Occasionally spontaneous crystallization occurs, but usually the solution remains supersaturated for many hours. It is seeded with a suspension of crystals in ethanol—this is important, for when dried the crystals quickly lose their solvent of crystallization and become amorphous. After some hours the suspension is gradually cooled, a convenient way of accomplishing this in the absence of a slowly cooling thermostat being to transfer the container (well wrapped, or preferably in a Dewar jar) to the incubator at 24°, to room temperature, and then to the refrigerator, at least 2 days being allowed for the transition.

Crystals can equally well be obtained from ethanolic solutions containing more water, but the initial concentration of micrococcin must then be appropriately increased.

Occasionally an amorphous gel is obtained in spite of slow cooling of a slightly supersaturated solution. In such a case the gel, which has positive thixotropic properties, is filtered off from the mother liquor as completely as possible and redissolved in ethanol, a crop of crystals being almost always obtained.

The mother liquor from which crystals had been removed still contained active material, but little or none of this was obtained in crystalline form in spite of further chromatography and repeated deposition from slowly cooled, seeded ethanolic solution. It separated as a compact brown granular solid which tended to adhere firmly to the walls of the container. In antibacterial activity, fluorescence, solubility in ethanol-water mixtures and in other solvents, melting point behaviour, and inactivity against a staphylococcus made resistant to micrococcin, it behaved like nearly pure micrococcin. The possibility that it is not identical with micrococcin cannot be ruled out, and it may or may not be significant that the chromatogram was not quite typical of that obtained in the first stage of purification, in that the purple fluorescent zone was less marked, and more of the activity appeared to occur in the top, less fluorescent, brown, band.

Chemical properties

Elementary composition

A finely divided, twice recrystallized, well mixed sample, pure white in colour, was found to contain C, H, N and S; halogens and P were absent. Some of the material was submitted to Drs Weiler and Strauss for C, H, N, S, *C*-Me and OMe determinations, with the following results: N, 13·6; S, 15·9; OMe, nil; *C*-Me, 6·9; C, 49·0; H, 4·6; C and H residue, 2·4 %.

In view of the residue, a sulphated ash determination of the same sample was asked for, a value of 3·77 being obtained. Attempts were made by the authors to obtain ash for further examination, but after much work and several consultations with, and further analyses by, Dr F. B. Strauss, the conclusion was reached that no true ash, other than traces of alumina and a trace of volatile ash, was present, but that during combustion an unusually stable organic residue might be left. This remained even after a glow had passed through the solid in an atmosphere of O_2; it was black in colour, and contained C and H. Dr Strauss kindly examined the emission spectrum of the same sample of micrococcin with Cu electrodes, and reported that there was no indication of the presence of any metal other than alumina.

Two more analyses by Drs Weiler and Strauss on the same sample of micrococcin gave the following values: (1) C, 49·5; H, 4·6; residue, 1·5; (2) C, 49·3; H, 4·6; no residue. $C_{25}H_{28}O_6N_6S_3$ requires: C, 49·7; H, 4·6; N, 13·9; S, 15·9 %; but owing to the special difficulty of combustion the analytical figures are probably subject to errors greater than usual.

Molecular weight

By Barger's method in 80 % ethanol + 20 % water with azobenzene as reference compound, the molecular weight appeared to be slightly greater than 2170 and definitely less than 2720. However, the solutions were of the order of 0·01-M and thus near the lower limit of workability of the method.

Behaviour to heat

Melting point. The material darkens and sinters at 222–228°, but does not have a sharp melting point.

On heating alone the powder softens to a yellowish syrup which darkens and bubbles, emitting a distinct but indefinable odour. On further heating yellow fumes are evolved and a tar condenses on the cooler parts of the tube, while the odour of ammonia can just be detected in the alkaline vapours. As the tar is driven off the black residue becomes hard and brittle.

On heating with lime ammonia is evolved.

On treating with a drop of sulphuric acid micrococcin chars at once, and on gently warming the black treacly mixture swells up and bubbles, as would a carbohydrate. There is a strong smell of acetic acid at first, which is later replaced by an equally strong smell of acetamide.

Tests for reactive groups

As shown above, methoxyl groups were absent, and the material contained 6·9 % of *C*-Me.

Ethanolic ferric chloride gave no appreciable colour with an ethanolic solution of micrococcin, and the nitrous acid test for phenols (Feigl, 1946) was also negative. Sodium nitrite in contact with a glacial acetic acid solution of micrococcin for 30 min. at room temperature before treatment with urea and neutralization caused no change in either fluorescence or antibacterial activity and no gas was evolved. Negative reactions were obtained with Schiff's and Millon's reagents and with ninhydrin. A hydrolysate prepared by digesting a 1 % solution of micrococcin in equal parts of concentrated hydrochloric acid and glacial acetic acid for 21 hr. in a sealed tube at 100–110° gave a clear solution while hot, but when cold a small amount of creamy coloured flocculent solid appeared. A ninhydrin-reacting substance was found in the hydrolysate, but this was identified as ammonia by the formation of ammonium picrate in the Conway diffusion apparatus. Ammonia present in the hydrolysate accounted for 24·2 % of the total nitrogen in micrococcin.

When solid micrococcin was added to a mixture of iodine and sodium azide (Feigl, 1946; test for —SH and =S) there was no trace of evolution of gas, but the particles of micrococcin stained brown. On adding an ethanolic solution of micrococcin to the reagent some of the micrococcin was precipitated, but there was still no evolution of gas.

Bromine was only slowly decolorized by dilute solutions of micrococcin, but both fluorescence and antibacterial activity were reduced. When micrococcin in solution in aqueous ethanol was treated with zinc and hydrochloric acid there was a pronounced smell of hydrogen sulphide and after 0·5 hr. both fluorescence and antibacterial activity had fallen to about 1 % of their original value.

Physical properties

Crystalline form

The crystals consist of fine long silky needles—sometimes extremely long in relation to their thickness—nearly always aggregated into fans, sheaves, or little balls. They are birefringent, showing crossed extinction, and in the dry state lose solvent of crystallization and birefringence and become pseudomorphs. On the other hand, in bulk the ethanol of crystallization is lost only slowly, even over calcium chloride in a vacuum desiccator, and partially dried crystalline material can apparently reabsorb ethanol from the vapour phase.

Solubility

In water. Although the microbiological assay of a saturated watery solution presented no difficulty the preparation of a just saturated solution was by no means easy. At room temperature values of less than 0·2 to more than 1·5 μg./ml. were obtained, and it was impossible to decide whether with the lower values the solution was not saturated, or whether with the higher values some particulate matter was present. None of the methods of preparing a saturated solution which were tried was satisfactory, but the solubility of micrococcin in water at room temperature is believed to be about 1 μg./ml.

In aqueous ethanol. The solubility was found to depend very markedly on the proportions of water and ethanol in the solvent, reaching a maximum at

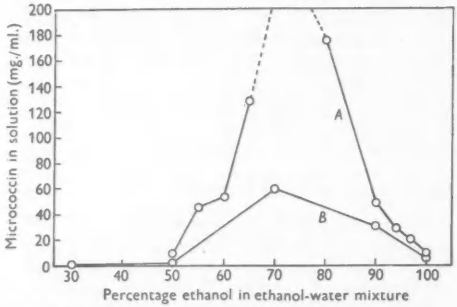

Fig. 2. Solubility of micrococcin in ethanol-water mixtures at 25±1° (curve *A*) and 2·8±0·1° (curve *B*).

about 75% ethanol, and falling steeply on either side. The actual values given in Fig. 2, which summarizes a series of experiments, are subject to several minor errors:

The process of saturation was carried out in constant temperature rooms where the suspension could be centrifuged and a sample of supernatant taken without materially changing the temperature, but the temperature in the rooms was seldom exactly the same at successive samplings. With the more dilute solutions it was found that 2 hr. or more might be required to reach equilibrium and with the stronger solutions, whose viscosity rose until at about 200 mg./ml. the fluid would hardly flow when the tube was inverted, very much longer might be required. Occasionally, with the stronger solutions, the whole might set to a stiff gel (due, perhaps, to a slight fall in temperature) which could not be centrifuged down. Then again, each time a sample was withdrawn, the composition of the solvent might change slightly by evaporation of ethanol and absorption of water, and owing to the steepness of the curve cause a significant change in solubility actually found.

In chloroform. In this solvent, whether carefully dried, or wet, solid micrococcin dissolved very rapidly. The excess solid became gelatinous and swelled until substantially the whole volume of the solution was occupied by a gel which was more or less rigid depending on the amount of solid taken. This interfered with sampling and it was not possible to obtain reproducible results, but at 20° the solubility in either dry or wet chloroform appeared to be about 8 mg./ml.

Ultraviolet fluorescence

Dry micrococcin had a definite but not especially powerful purple fluorescence in ultraviolet light. In solution it had a powerful purple fluorescence in all solvents tested, except in phenol. (This was due to

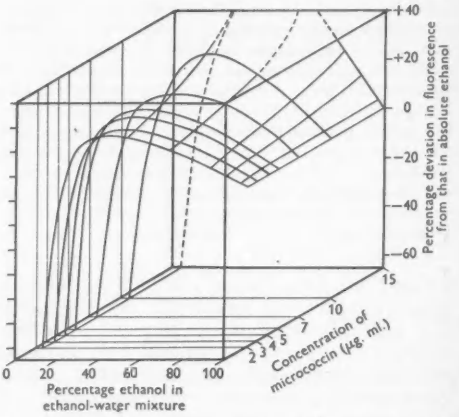

Fig. 3. The effect of the composition of the solvent on the expected fluorescence of micrococcin solutions, for different concentrations of micrococcin. (From Heatley, Kelly & Smith, 1951.)

quenching of fluorescence, not destruction of micrococcin, for removal of the phenol with ether restored the fluorescence.) The quantitative observations of fluorescence were made in the Beckman model DU spectrophotometer fitted with fluorimeter attachment, and were expressed as apparent concentration of micrococcin, as read from calibration curves (Heatley, Kelly & Smith, 1951).

Effect of temperature. There was no difference by eye between the fluorescence of ethanolic solutions at +2° and +20°, but at boiling point the fluorescence was slightly diminished.

Effect of dissolved oxygen. The fluorescence (measured by the Beckman apparatus) remained unchanged after H_2 had been bubbled through an ethanolic solution of micrococcin for 40 min. It was also unchanged on shaking with air or on adding H_2O_2.

Effect of water. The intensity of fluorescence increased as the proportion of water to ethanol was increased, reaching a maximum at about 60% of water and thereafter falling almost to zero. The magnitude of the effect depended on the concentration of micrococcin, and Fig. 3 shows the relation

between the three quantities—composition of solvent, concentration of micrococcin, and difference in fluorescence from that in pure ethanol.

Effect of acid. Hydrochloric acid, up to 0·2 N, did not affect the fluorescence of an ethanolic solution.

Effect of alkali. Alkali (0·03 N, final concentration) was observed to reduce the fluorescence of ethanolic solutions almost to zero, but on neutralizing and removing the precipitated NaCl it was completely restored, even after having been kept alkaline for several hours. The antibacterial activity was also unaffected by such treatment. When water was present the effect of the alkali was less marked, and in experiments in 98·5, 80 and 50% ethanol the fluorescence was reduced to 14, 33, and 51% of its original value, respectively. After neutralization over 90% of the original fluorescence was restored and no diminution of antibacterial action was detected.

When the concentration of alkali was altered the diminution of fluorescence changed also. In one experiment (in 98% ethanol) the following results were found:

Final concentration of alkali (N)	Fluorescence (% of original value)
0	100
0·01	29
0·02	17
0·03	11
0·05	7·5
0·1	4
0·2	2

No attempt was made to determine the pH of these ethanolic solutions.

Effect of sunlight. When a drop of micrococcin solution was placed on a filter paper and allowed to dry, the spot was invisible by daylight, but had a pure purple fluorescence in ultraviolet light. When the paper was exposed to sunlight or to ultraviolet light the spot became yellowish and the purple fluorescence in ultraviolet light was replaced by a less bright, pale-yellow fluorescence. The purple fluorescence disappeared completely after 5 min. in strong sunlight, but on adding a drop of ethanol to the filter paper the solvent immediately acquired a brilliant purple fluorescence, suggesting that only the surface of each micrococcin particle was initially affected.

The change appeared to be a light-activated oxidation, for no yellowing occurred even after several hours' exposure to sunlight if the paper were sealed in an evacuated tube. Similarly, the appearance and fluorescence of the spot were unchanged for weeks if kept in air in the dark. On extending the exposure to ultraviolet light or sunlight the yellowish fluorescence very gradually became stronger, while the intensity of the purple fluorescence washed out of the paper by ethanol became less, in parallel with the antibacterial activity. The yellow spot was not washed out of the filter paper by ethanol, water, or dilute acid; its fluorescence was reversibly quenched by alkali. The same change took place, though much more slowly, when an ethanolic solution of micrococcin was exposed to light. There was no evidence that, once started, the reaction would proceed in the absence of light.

Ultraviolet absorption

The absorption spectrum of twice crystallized micrococcin at 20 μg./ml. in ethanol is shown as curve *B* of Fig. 4. That of Su's (1948) purest preparation at the same concentration has been superimposed as curve *A*. The curves are closely similar, and it can be deduced that Su's preparation was about 86% pure, in terms of the crystalline material.

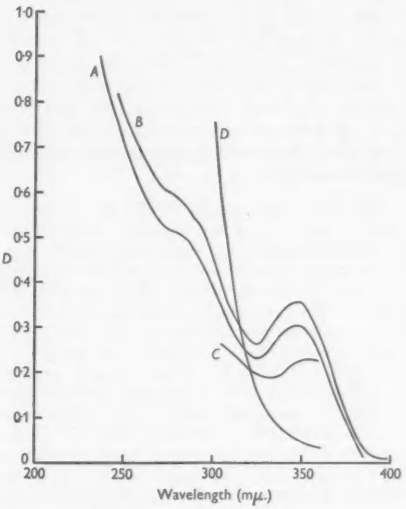

Fig. 4. Ultraviolet absorption spectra of micrococcin, at 20 μg./ml. in ethanol. Curve *A*, as reported by Su (1948) for his purest preparation. Curve *B*, twice recrystallized micrococcin. Curve *D*, copper, 1 in 10^4 (as sulphate) in ethanol. Curve *C*, twice recrystallized micrococcin, 20 μg./ml., plus copper, 1 in 10^4, after subtraction of curve *D*.

At a wavelength of 345 mμ. Beer's Law was found to be obeyed between concentrations of 5 and 60 μg./ml. in ethanol. The effect of traces of copper on the absorption are discussed below.

Infrared absorption

This was kindly examined by Dr L. N. Short. The presence of strong bands at 1657 and 1536 cm.$^{-1}$ in the spectrum of the solid suggested that the compound contains an amide group. In dilute solution in chloroform a band was given at 3384 cm.$^{-1}$, as would be expected for a mono-substituted amide. Many bands were given in the region 700–1400 cm.$^{-1}$, but no conclusions could be drawn from these regarding the structure of the compound.

Optical rotation

In 90% ethanol a twice-crystallized and carefully dried preparation, at a concentration of 50 mg./ml., had $[\alpha]_D^{21°} = +116° \pm 1°$.

The effect of heavy metals

Su (1947, unpublished) incubated small pieces of metallic copper, iron, and zinc for 24 hr. in an active crude culture fluid of the micrococcus and found no diminution of antibacterial activity. However, it has been found that traces of copper salts profoundly depress the fluorescence of ethanolic solutions of micrococcin, while tin, ferrous, and ferric salts have a definite though less marked effect. Attempts to find a stoicheiometric relation between copper and micrococcin showed that the effect was complicated: from several experiments the following points emerged:

(1) The main diminution of fluorescence on adding Cu occurred in less than 1 min.—possibly instantaneously. There was then a further small decrease during the next few hours. (2) Cu (as sulphate) at 0·1 p.p.m. gave an observable diminution of fluorescence; at 1 p.p.m. over 50% of the fluorescence of an ethanolic solution containing 20 μg. of micrococcin per ml. was abolished at once; but the fluorescence was not abolished completely, even with a copper concentration of 100 p.p.m., the highest practicable. (3) H_2O_2 had no effect on the fluorescence of ethanolic micrococcin alone. When added to micrococcin + copper some of the lost fluorescence was occasionally restored. Shaking a micrococcin-copper solution with air caused small variable changes in fluorescence. Prolonged bubbling of hydrogen through the solution had no effect. (4) The addition of BAL (2:3-dimercaptopropanol) to a copper-micrococcin solution restored some but not all of the fluorescence. This restoration was at first rapid—perhaps instantaneous—and there was sometimes a further slight increase in fluorescence during the next few minutes. Additional BAL had no effect. Copper mixed with BAL before adding to micrococcin solution had no effect on the fluorescence. (5) The effects of oxine or thioglycollate could not be followed because their coloured complexes with Cu interfered with the fluorescence of micrococcin. (6) The ultraviolet absorption of micrococcin was also modified by copper, as shown in Figs. 4 and 5, the change being in degree rather than in character. This change was also partly reversed by BAL, but owing to its smallness and to the strong absorption of mixtures of BAL and Cu, quantitative deductions were subject to a somewhat large error. However, it appeared that when BAL was added to a micrococcin-copper mixture the percentage restoration of fluorescence and of absorption (at 345 mμ.) was the same.

We are indebted to Dr W. S. Metcalf for suggestions and advice on this part of the work. He considered that the evidence available favoured the supposition that micrococcin formed a compound containing Cu.

The effect of copper on the antibacterial action of micrococcin. Some preliminary experiments were carried out to test whether the antibacterial action of micrococcin was modified by copper, as were its fluorescence and absorption spectrum. The conflicting results led to the reflexion that as the effect of copper was reversible, its effect on antibacterial action would be difficult to study, for the following reasons: (1) The fluorescence and absorption are measured in ethanol which must, for antibacterial assay, be largely evaporated. This would change the concentration of the volatile BAL, if present, and would probably cause precipitation of copper-micrococcin and copper-BAL complexes which might not redissolve in watery media. (2) The constituents of the medium would be expected to bind copper and alter its effective concentration. (3) The copper might itself inhibit the growth of the test organism. For these reasons further investigation of the effect of copper on the antibacterial effect of micrococcin was abandoned.

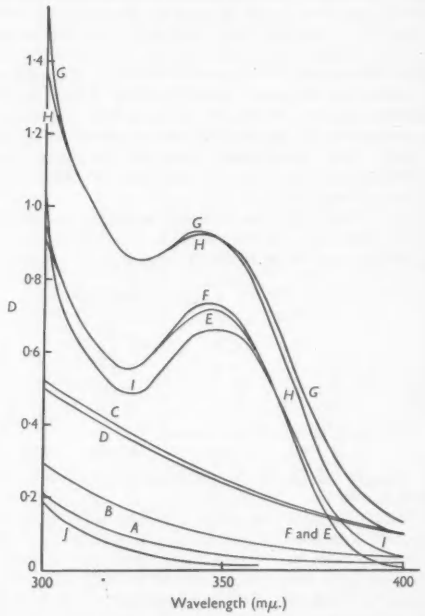

Fig. 5. Ultraviolet absorption spectra of micrococcin, copper sulphate, and BAL, and of mixtures of them, in ethanol. Copper (1 in 10^8) alone (curve *J*) and with 0·2, 0·5, 1 and 5 equivalents of BAL (curves *A–D*). Curves *E* and *F*, micrococcin 40 μg./ml. plus about 8 equivalents of BAL. Curves *G* and *H*, micrococcin, 40 μg./ml., plus copper, 1 in 10^8, plus 1 equivalent (curve *G*), and 5 equivalents (curve *H*) of BAL. (Approx. molar ratio of micrococcin:copper:BAL is 1:8:8 in curve *G* and 1:8:40 in curve *H*). Curve *I*, curve *G* minus curve *C*.

SUMMARY

1. The antibiotic micrococcin, extracted by Su (1948) from *Micrococcus*sp. (N.C.T.C. no. 6571), has been crystallized. The mother liquor may contain another antibiotic closely similar to micrococcin.

2. Micrococcin is almost insoluble in water. In ethanol-water mixtures its solubility rises to >150 mg./ml. at 70–80% ethanol, then falls to less than 10 mg./ml. in absolute ethanol.

3. It decomposes at 222–228°. $[\alpha]_D^{21°} = +116°$. It has a broad absorption in the ultraviolet and many bands in the infrared. An ethanolic solution has a strong purple fluorescence in near-visible ultraviolet light.

4. Both fluorescence and ultraviolet absorption are reduced by traces of copper and partly restored by BAL.

5. The molecular weight appears to be somewhat over 2000, and elementary analysis gave C, 49–49·5; H, 4·6; N, 13·9; S, 15·9%. It gives a positive ninhydrin reaction only after acid hydrolysis, but this seems to be due to ammonia, not amino-acids. Nothing is known of its chemical nature.

We are indebted to Miss E. Page and Miss S. Long for valuable technical assistance throughout this work; to Mr B. K. Kelly, Director of the Medical Research Council Antibiotics Research Station, Clevedon, and his staff, for most of the micrococcin; and to Drs Ella M. Barnes and Kathleen Crawford and their assistants Miss C. King and Mr D. Jackson for some of the early fermentations. One of us (H.M.D.) is indebted to the Australian National Health and Medical Research Council for a personal grant.

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Metabolic Effects of Electrical Stimulation of Mammalian Tissues *in vitro*

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It has been found that electrical stimulation of slices of guinea pig brain cortex *in vitro* gives rise to large increases in the rate of oxygen consumption and aerobic glycolysis (McIlwain, 1951; McIlwain, Anguiano & Cheshire, 1951).

In order to test the scope of the new method, as to whether the response to electrical stimulation is confined to nerve tissue or is a property of other tissues, the technique has now been applied to rat diaphragm, and guinea pig lung and kidney slices.

Isolated whole frog muscle responds to electrical impulses by increased oxygen consumption which is several times that of the resting muscle (Meyerhof & Schulz, 1927; Fenn, 1927; Gemmill, 1934). Also Millikan (1937) has shown that *in vivo* cat muscle, during active contraction, can respire at a rate nearly twenty times that of the muscle at rest. Little work has been done to see what response is made to electrical impulses *in vitro* by other mammalian tissues.

METHODS

Guinea pig kidney cortex and lung slices, 0·35 mm. thick, were cut by means of a Stadie-Rigg's cutter. The thickness of slices was less than the maximum limit stated by Warburg (1930) and Field (1948) as being allowable for full oxygenation. Guinea pig-brain cortex slices were cut as described by McIlwain & Grinyer (1950). The diaphragms of young rats of about 100 g. were dissected out and the peripheral muscle portions used. According to Cohen (1949*a*) the diaphragms from animals of this size have a thickness of about 0·3 mm. The procedure in weighing tissues was that described by McIlwain (1951).

Krebs-Ringer phosphate solution, as described by Cohen (1949*b*), was used with the concentration of calcium reduced by half to prevent the formation of a precipitate. Glucose was added to give a concentration of 0·1% (0·0056M). Media were oxygenated at 37°.

The manometer flasks, of conical shape, were of about 18 ml. capacity; to some of them lead-in wires were inserted (type *A*, McIlwain, 1951). The tissues were fixed in small plastic and silver gauze tissue-holding electrodes (type *B*,

McIlwain, 1951). In most of the experiments, the control tissues were fixed in similar electrodes. The centre well contained 0·1 ml. 5N-KOH and paper to absorb CO_2. An experiment was made in stages as follows: (1) a preliminary equilibration period of 10–15 min.; (2) measurement of resting O_2 uptake over a period of 20–30 min. (period A); (3) measurement of O_2 uptake while the tissues were exposed to electrical impulses (period B); this treatment was omitted in the control experiments. The applied voltage was 2·5 V. (as measured by a moving coil meter) supplied by a variable transformer run from the 50-cycle a.c. mains. The current was about 10–25 ma. The greater proportion of this current was short circuited through the saline and did not pass through the tissue: this was tested in one instance by the currents with and without the saline present. The currents were respectively 13 and 0·1 ma. In a few experiments alternating pulses from a 2 or 4 μF. condenser, charged from a 12 or 24 V. battery, as described by McIlwain (1951), were used as an alternative form of impulse. The time constant of discharge was between 1 and 2 msec.

Lactate was measured by the method of Barker & Summerson (1941).

RESULTS

Normal respiration. The means of all initial (period *A*) rates of oxygen uptake are given in Table 1. These include the values for the controls which were treated in exactly the same way as the test material in this initial period.

All tissue weights quoted are the initial wet weights. These have been used in preference to the final dry weights, for there is evidence (Bach, 1944; Cutting & McCance, 1946) that leaching of the solid matter of tissue slices occurs in physiological salines. In order to compare the rates of oxygen consumption with those of other workers who expressed their results upon post-experimental dry weight of tissue, the dry/wet weight ratios for fresh slices have been determined. The values are: brain cortex, 0·16; lung, 0·15; kidney cortex, 0·20; diaphragm, 0·26. The corresponding rates of oxygen uptake are shown in Table 1. It is seen that they are

Table 1. *Rate of oxygen consumption during initial period*

(Incubation at 37° in O_2 of slices suspended in modified Krebs-Ringer phosphate solution.)

Animal	Tissue	No. of animals	No. of determinations	Initial oxygen consumption (μmol./g. wet wt./hr.)	Initial oxygen consumption (μl./mg. dry wt./hr.)	Values recorded by other authors (μl./mg. dry wt./hr.)	Reference
Guinea pig	Brain cortex	4	12	73·1±4·9*	10·2	12·9	Wollenberger (1947)
						11·7	Edson & Leloir (1936)
						11·0	Jowett & Quastel (1937)
Guinea pig	Lung	11	52	40·5±1·6	6·0	7·0	Wollenberger (1947)
						6·1	Simon, Potts & Gerard (1947)
						6·1	Barron, Miller & Bartlett (1947)
Guinea pig	Kidney cortex	6	36	123·9±5·2	13·9	16·8	Wollenberger (1947)
						19·0	Edson & Leloir (1936)
						(μmol./g. wet wt./hr.)	
Rat	Diaphragm	12	23	68·8±2·9	5·9	75·9	Walaas & Walaas (1950)
						46·5	Gilmore & Samuels (1949)

* Standard error of the mean.

Table 2. *Oxygen consumption on exposure to* 2·5 V. *a.c.*

Tissue slices		No. of determinations	Mean O_2 consumption (μmol./g. wet wt./hr.) Initial period	On exposure to current	Mean change (%)	Statistical significance of change caused by electrical stimulation
Brain cortex:	*a*, control tissue	6	72·0	64·4	− 8·0±5·2*	S ($P<0·01$)
	b, exposed tissue		74·2	112·0	+51·0±9·9	
Lung:	*a*, control tissue	26	40·5	41·0	+ 2·8±3·1	S ($P<0·01$)
	b, exposed tissue		40·5	47·1	+18·5±4·6	
Kidney cortex:	*a*, control tissue	18	120·0	112·5	− 6·0±1·4	NS
	b, exposed tissue		127·8	120·8	− 5·8±1·4	
Diaphragm:	*a*, control tissue	12	64·4	60·7	− 5·2±2·4	S ($P<0·01$)
	b, exposed tissue		73·4	101·8	+38·6±6·6	

* Standard error of the mean. S, Significant. NS, Not significant.

Table 3. *Alternating current on respiration of lung slices*

(Slices in modified Krebs-Ringer phosphate solution at 37°, exposed to 2·5 V. Figures in corresponding positions in the table refer to the same tissue slice. The variability as between different slices is probably due to the difficulty of obtaining a reliable wet weight of the spongy lung slice.)

Respiration during initial period (μmol./g. wet wt./hr.)				Respiration on exposure to impulses (μmol./g. wet wt./hr.)			
65	24	32	20	85	42	42	23
42	37	45	45	32	38	53	48
32	32	50	38	39	30	47	49
40	40	33	21	57	47	47	33
44	37	46	45	60	54	57	50
41	48	61	43	37	44	60	51
39	51			49	49		
Mean 40·5				Mean 47·1			

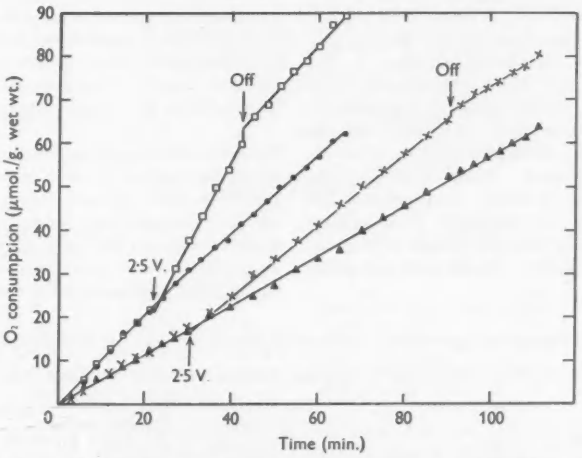

Fig. 1. The effect of 2·5 V. alternating current on the O_2 consumption of rat diaphragm and guinea pig-lung slices in modified Krebs-Ringer phosphate media. The instantaneous displacement on applying and switching off electric current is due to its heating effect (see McIlwain, 1951). Diaphragm: control, ●; exposed, □. Lung: control, ▲; exposed, ×.

rather lower than the values recorded by others. This may perhaps be accounted for by the reduction in weight due to leaching. There was found to be only a small difference between the oxygen consumption of a tissue held in the electrode and that of the same tissue in free suspension. Four experiments with diaphragm showed no appreciable difference under the two sets of conditions. In six experiments with brain cortex slices, the oxygen consumption of the tissue held in the electrode was 3 % greater, and with lung slices 12 % greater than that of the freely suspended tissue.

Influence of electrical impulses on respiration. The respiration rates of the tissues during the passage of electrical impulses are shown in Table 2. The statistical significance of observed increases over the normal values has been estimated by the 't' test.

The respiration of brain cortex slices showed a significant increase ($P < 0·01$) over the control value during exposure to impulses. The same was true for diaphragm. The experiments with lung slices, when examined as a whole, also showed a significant increase ($P < 0·01$). However, in contrast to brain cortex and diaphragm, which responded to the electrical impulses in every case, seven of the twenty-six lung slices showed no increase of respiration during the passing of the current, while with the others there was a graduation of response to a maximum of 75 %.

The individual results are given in Table 3. This variability of the response of lung is thought to be due to the presence of varying amount of muscle in the slices, which were cut from the apex at different depths. Kidney cortex, a tissue which contains no

muscle fibres, gave no response to electrical impulses; the respiration of both the experimental and the control slices fell by 6 % of their initial rate during period B.

McIlwain (1951) has reported that stimulation caused no permanent increase in the rate of oxygen consumption of brain cortex slices. In the present experiments the rate was similarly found to return nearly to its original value in those cases where a post-stimulation period was run. Examples for diaphragm and lung are shown in Fig. 1.

The alternative form of stimulation, by condenser discharge, was used in some experiments. In two cases lung slices showed no response, but in a third a 36 % increase in respiration was recorded. The respiration of diaphragm increased by 33 % as a result of condenser impulses. Kidney slices showed no response. These few experiments confirm the results obtained with alternating current.

Glycolysis. McIlwain (1951) has found that stimulation of brain tissue markedly increases its aerobic glycolysis. Therefore, it was decided to make similar tests with the different tissues used for the experiments on respiration. In order to facilitate the detection of any increase in glycolysis, the period of stimulation was arranged to be greater than half the total time that the tissues were in the manometric vessels. The results are given in Table 4.

Table 4. *Increase in lactic acid production by tissues on exposure to electrical impulses*

(All tissues suspended in modified Krebs-Ringer phosphate solution, pH 7·4, under O_2 at 37°, exposed to 2·5 V. a.c.)

Tissue	No. of pairs of determinations	Total experimental time (min.)	Period of exposure (min.)	Mean production of lactic acid by control slices (μmol./g. wet wt./ experimental time)	Mean production of lactic acid by exposed slices (μmol./g. wet wt./experimental time)	Mean change (%)	Significance of increase
Brain cortex	4	90–95	50–60	45·0±6·3	70·2±4·0	+56	S ($P<0·05$)
Lung	13	95–100	55–60	40·5±4·0	38·2±7·3	−6	NS
Kidney cortex	7	90–95	50–60	41·6±7·0	46·6±4·2	+13	NS
Diaphragm	9	80–90	50	22·6±4·3	33·1±4·8	+47	S ($P<0·05$)

S, Significant. NS, Not significant.

Lactic acid production by the control portions of diaphragm was greater than that reported by Gilmore & Samuels (1949), and Walaas & Walaas (1950), but in tissue slice experiments, lactic acid production can vary greatly with tissue/fluid ratios and with the duration of the experimental period. The stimulating current produced further amounts of lactic acid; the percentage increase was 47 %. The production of lactic acid by the control slices of kidney cortex was higher than had been expected on the basis of earlier observations of Dickens & Šimer (1929) who found that kidney cortex produced only traces of lactic acid under aerobic conditions. They stated that aerobic glycolysis can be caused in tissues due to slight mechanical or other damage without affecting the rate of respiration. In the present experiments the tissues were handled much more than those used by Dickens & Šimer, and this may account for the present high rate of glycolysis. A comparison between slices of kidney cortex suspended freely in the medium and similar slices held in electrodes, showed no difference in the rates of lactic acid formation. Thus it seems that the electrodes do not increase production of lactic acid by damage or by preventing adequate gas exchange. Kidney cortex slices showed no significant change in lactic acid production as the result of electrical impulses. Lung slices also were found not to change their aerobic glycolysis on stimulation. The rate of formation of lactic acid by brain cortex slices in control experiments as well as the increases produced by stimulation are within the range reported by McIlwain *et al.* (1951).

DISCUSSION

From the above experiments it can be seen that there are definite metabolic responses to electrical stimulation *in vitro* by both brain and diaphragm; a smaller response by lung and no response by kidney cortex. The lack of response by kidney suggests that the positive responses of other tissues are not due to some general reaction of the electric current. Curtis & Cole (1947) state that most organs of the body are not harmed by even a prolonged application of an alternating electric current and some organs, for example liver, show no measurable reaction to such a current. The failure of kidney to respond in the present experiments may have been due to two causes; either the tissue was totally unresponsive, or the impulses used were not suitable. However, impulses with various characteristics have been used without success, and it appears likely that the tissue was unresponsive, at least by the criteria so far used to measure response.

It would seem that, apart from brain tissue, a metabolic response to electric impulses occurs only

if muscle fibres are present. Diaphragm is a muscular tissue and lung contains muscle fibres while kidney contains none. This is further borne out by some preliminary experiments using heart slices, where a response was found, and liver or testis which showed none.

The oxygen consumption of rat diaphragm has also been increased by the action of chemical stimulants. Gordon & Heming (1944) found that injection of thyroxine into rats raised the oxygen consumption of the excised diaphragm by 29% from their normal respiration level of 5·6 μl. oxygen/mg. dry weight tissue/hr. A similar response was obtained by Gemmill (1947) on incubating rat diaphragm with theobromine derivatives. Caffeine, for example, raised the respiration by 29% from a basal rate of 53 μmol./g. wet weight tissue/hr. In assessing the results of the present experiments with rat diaphragm these results are of value in showing that under somewhat similar conditions to those used here, rat diaphragm is able to respond to several forms of stimulation.

SUMMARY

1. Tissue slices of guinea pig-brain cortex, kidney cortex and lung, and portions of rat diaphragm have been exposed to fluctuating electric currents *in vitro*.

2. These caused a mean increase of 51% in the rate of brain cortex respiration and an accompanying increase of 56% in aerobic glycolysis.

3. The mean increase in the respiration of diaphragm was 39% with an increase of 47% in aerobic glycolysis.

4. The impulses increased respiration of lung slices by 19%, but gave no increase in aerobic glycolysis.

5. No response to the impulses was obtained with kidney slices.

I wish to thank Dr H. McIlwain for the helpful advice and criticism he has given during the course of these experiments and to Miss S. Russell for her technical assistance. In addition, I wish to thank Mr D. K. Hill for his comments on the manuscript.

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The L-Amino-acid Oxidase of *Neurospora*

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Bender, Krebs & Horowitz (1949) and Bender & Krebs (1950) reported that *Neurospora crassa* can produce an L-α-amino-acid oxidase in addition to the D-α-amino-acid oxidase previously described by Horowitz (1944). The L-amino-acid oxidase was present not only in the mycelium but also in the clear culture fluid, whilst the D-enzyme only occurs in the mycelium. The quantity of the L-enzyme formed was found to be dependent on the composition of the medium and to vary from strain to strain. The present paper is an extension of preliminary work by Dr A. E. Bender and is concerned with the factors influencing the production of the L-amino-acid oxidase, and with the properties of the enzyme, including the nature of the prosthetic group. A somewhat similar study has been simultaneously carried out by Thayer & Horowitz (1951) in the Kerckhoff Laboratories of Biology, Pasadena, U.S.A.

EXPERIMENTAL

Strains. The twenty strains of *Neurospora* used are shown in Table 1.

Conditions of culture. The basal medium was essentially that of Horowitz & Beadle (1943). It contained (final concentrations) 2% sucrose, 0·5% ammonium tartrate, 0·1% NH_4NO_3, 0·1% KH_2PO_4, 0·01% NaCl, 0·05% $MgSO_4.7H_2O$, 0·01% $CaCl_2$ and the following quantities of trace elements per litre: $Na_2B_4O_7$, 0·05 mg.; Na_2MoO_4, 0·04 mg.; $FeSO_4.7H_2O$, 1 mg.; $CuSO_4.5H_2O$, 0·4 mg.; $MnSO_4.7H_2O$, 0·1 mg. and $ZnSO_4.7H_2O$, 9 mg. Biotin, casein hydrolysate (low vitamin casein supplied by Genatosan Ltd., and hydrolysed according to McIlwain & Hughes, 1944) or other substances were added as indicated in the text. The culture flasks, either conical flasks, Roux bottles or the mould culture flasks of Clayton, Hems, Robinson, Andrews & Hunwicke (1944), were filled with liquid medium to a depth of about 1 cm. and autoclaved for 15 min. at 15 lb. pressure. Conidia from subcultures grown on agar slopes were used for inoculation except with strain CMI 3411 which did not regularly sporulate; it was propagated by transferring about a milligram of wet mycelium from subcultures grown on 10 ml. liquid medium in 25 ml. conical flasks. The growth temperature was 25°.

Determination of oxidase activity. O_2 consumption was determined in Warburg manometers of about 25 ml. volume at 30° and pH 8·3 in 0·033 M-pyrophosphate with a total fluid volume of 4·5 ml. The gas phase was O_2 and the centre well contained 0·2 ml. 2 N-NaOH and a roll of filter paper. The enzyme and substrate were placed in separate compartments and mixed after thermal equilibrium was established. In testing for the L-amino-acid oxidase, the substrate was L-phenylalanine ($1·125 \times 10^{-5}$ g.mol.). As there was no D-oxidase in the culture fluid, the equivalent amount of DL-phenylalanine could be used. In the later stages of purification, an extract of red blood cells (equivalent to 0·02 ml. blood) was added to supply catalase. In the case of the culture fluid or preparations obtained therefrom the rate of O_2 consumption was constant at least during the period of 5–25 min. after mixing unless more than 40 units of activity were present. The activity was calculated from this constant rate; one unit being defined as the amount of enzyme causing 1 μl. O_2 to be taken up in 10 min. The activity determined in this way was proportional to the amount of the enzyme preparation. The culture fluid did not absorb O_2 in the absence of added amino-acids.

Preparation of mycelia for measuring oxidase activity. The mycelia were briefly washed in distilled water. Extracts were prepared by grinding with sand and pyrophosphate buffer followed by centrifugation according to Horowitz (1944), and suspensions by disintegrating with 20 ml. water in a small metal cup fitted to the Waring blender. These preparations usually possessed a high endogeneous respiration which precluded the exact assessment of the oxidase activity. In contrast to other strains the endogenous respiration of CMI 3411 and CMI 17836 was low, usually much less than 10% of the steady rate of O_2 consumption after adding phenylalanine; the activity of the mycelial preparations was calculated as for the culture fluid correcting for the endogenous respiration which was determined in a separate manometer vessel.

Flavin-adenine dinucleotide (FAD) was prepared from yeast by the method of Warburg & Christian (1938), but omitting the final fractionation of the Ba salt. The preparation was freed from riboflavin phosphate by ascending paper chromatography using the solvent mixture *tert.*-butanol (60 vol.)–pyridine (25 vol.)–water (15 vol.). The paper was dried at 80° in the dark, the FAD band cut out and washed in ether. The flavin was eluted from the paper by water. The concentration of FAD in this solution was obtained from the light absorption at 450 mμ. assuming that the molecular extinction coefficient ($\epsilon_{450\,m\mu}$) is $1·13 \times 10^4$ (Warburg & Christian, 1938).

Riboflavin phosphate was obtained in aqueous solution by hydrolysing FAD with N-HCl at 100° for 10 min.

Apo-enzyme of kidney D-amino-acid oxidase was prepared according to Negelein & Brömel (1939). The protein component obtained after washing in 18% $(NH_4)_2SO_4$ solution at 0° was dissolved in 0·006 M-pyrophosphate buffer, pH 8·3; the pH of the solution was then 6·5. This material was stored at −10°.

Light absorption was measured in 10 mm. cells in a Beckman model DU quartz spectrophotometer.

Fluorescence measurements were made with exciting light of about 365 mμ. filtered from a mercury vapour lamp.

Non-dialysable N of enzyme preparations. The solution of enzyme was dialysed against running tap water until free from ammonium ions. 1·0 ml. was digested with 0·20 ml.

Table 1. *Strains of* Neurospora *examined*

Strain	Growth factor required (other than biotin)	Source
	N. crassa	
CMI 3411	—	Commonwealth Mycological Institute, Ferry Lane, Kew
CMI 17836 (Beadle 37401)	Inositol	
CMI 19421 (Beadle 4545)	Lysine	
CMI 24298 (Beadle 9185)	Aneurin	
CMI 31288 (Beadle 1633)	*p*-Aminobenzoic acid	
Beadle 25*a*	—	Dr N. H. Horowitz, California Institute of Technology, Pasadena, U.S.A.
Abbot *a* and *A*	—	Dr D. G. Catcheside, Botany School, Cambridge
Beadle 1*A*	—	
Cambridge *a* (a spontaneous mutant of Beadle 25*a*)	—	
Chilton *a*	—	
Emerson *a* and *A*	—	
Lindgren *a* and *A*	—	
31223–8	α-Amino-acids (see Fincham, 1950)	Dr J. R. S. Fincham, University College, Leicester
47305		
	N. sitophila	
CMI 34458	—	Commonwealth Mycological Institute
CMI 34457	—	
Beadle 299	Pyridoxine	Dr E. C. Barton-Wright, Whitbread and Co. Ltd., Chiswell Street, London, E.C. 1

50% H_2SO_4, 1% SeO_2 for 4 hr. in a test tube. The tube was cooled, 4·9 ml. water added and the solution mixed with a glass rod. Nessler's reagent (3 ml.) was added and the colour compared photometrically with standards.

Ammonia was estimated in the Parnas apparatus (Parnas & Heller, 1924).

Pyruvic acid was estimated by the carboxylase method (Krebs & Johnson, 1937).

RESULTS

Factors influencing the yield of L-*amino-acid oxidase*

As already shown (Bender *et al.* 1949; Bender & Krebs, 1950) the yield varies from strain to strain and with the supply of biotin. It also depends on the presence of casein hydrolysate and the use of stationary culture as opposed to shaken culture. The highest yields of oxidase were obtained with strain CMI 3411 in stationary liquid culture on a sucrose, ammonium tartrate, inorganic salts medium supplemented by casein hydrolysate and 0·2–0·3 μg. biotin/l.

Table 2. L-*Amino acid oxidase in culture fluid*

(*N. crassa* grown 14 days at 25° on basal medium with 0·3% (dry) malt extract and 0·01% (dry) yeast extract.)

Strain	Oxidase activity (units/ml.)
Abbot *A*	2·3
Abbot *a*	3·3
Cambridge *a*	8
Lindgren *A*	7·6
CMI 3411	16
CMI 17836	4·7

Strain differences. Seventeen strains of *N. crassa* and three of *N. sitophila* (Table 1) were examined. They were grown in 1 l. Roux bottles at 25° on the basal medium (100 ml.) containing 0·3% (dry) malt extract and 0·01% (dry) yeast extract (compare Bender & Krebs, 1950). After 14 days' growth, 3 ml. samples of the clear culture fluid and 2 ml.

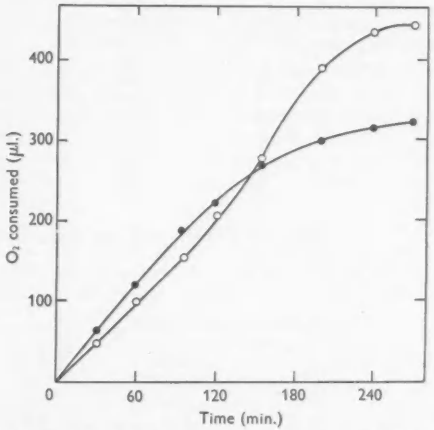

Fig. 1. Effect of L-phenylalanine on O_2 consumption by a mycelial extract (*N. crassa* strain Lindgren *a*). Manometer flasks contained: 2 ml. extract and 2 ml. 0·05 M-pyrophosphate buffer, pH 8·3. Side bulb: 0·1 M buffer (0·5 ml.) either alone (●) or with the addition of 1·88 mg. L-phenylalanine (○). Contents were mixed after 10 min. equilibration. Temp., 30°; gas, O_2.

samples of mycelial extract were examined for L-amino-acid oxidase activity. Activity was detected in the culture fluid from six strains of *N. crassa*, but not from the remaining eleven strains nor the three strains of *N. sitophila*.

With mycelial extracts from strains CMI 3411, CMI 17836, Abbot *a* and Lindgren *A* the oxidation of L-phenylalanine (1·88 mg.) began after a lag period of a few minutes and was complete in less than 45 min. With extracts from other strains the addition of phenylalanine at first reduced the rate of O_2 consumption compared with the control, but after 30–150 min. the oxygen consumed with added phenylalanine overtook that of the control (Fig. 1); the extra consumption rose to about 127 μl., corresponding to 1 atom of oxygen per molecule of phenylalanine. Whatever the initial effect of adding L-phenylalanine, about 127 μl. extra oxygen was eventually consumed by extracts from every strain, indicating the presence of the L-amino-acid oxidase.

The initial inhibition of the endogenous respiration by phenylalanine may be connected with the inhibition of the L-oxidase by high substrate concentrations (see later).

Duration of growth. *N. crassa* CMI 3411 was selected for further study because of its high oxidase activity. Yields of oxidase obtained on the synthetic basal medium plus biotin (0·25 μg./l.) were at least as high as those on the medium containing yeast and malt extracts. Maximum yields were obtained in 12 days (Table 3) on the synthetic medium with or without the addition of 0·2% casein hydrolysate. At this stage the total units of oxidase activity in the culture fluid were approximately the same as the total activity found in the mycelium.

Biotin supply. Bender *et al.* (1949) found that the yields were very much reduced with high concentrations of biotin (5 μg./l.) in the culture medium. This effect has been studied in more detail (Table 4). Optimal yields of oxidase were obtained with

Table 3. *Duration of growth and yield of* L-*amino-acid oxidase*

(*N. crassa* CMI 3411 grown at 25° on 40 ml. basal medium with 0·3 μg. biotin/l. in 250 ml. conical flasks.)

	Oxidase activity (units)			
	0·2% casein hydrolysate added to culture medium		No casein	
Days growth	Mycelial suspension	Culture fluid	Mycelial suspension	Culture fluid
4	700	200	530	130
6	1800	700	1040	400
9	2320	1700	1360	980
12	2550	2600	1500	1430
13	1700	2400	960	1330
14	900	2000	770	1030
16	630	1700	480	1000
19	500	1100	400	700

Table 4. *Effect of biotin and casein hydrolysate on the yield of* L-*amino-acid oxidase*

(*N. crassa*, CMI 3411 grown 10 days at 25° on 20 ml. medium in 50 ml. conical flasks. Dry weight determined on duplicate culture.)

Additions to growth medium			Oxidase activity (units)		
Biotin (μg./l.)	Casein hydrolysate (%)	Mycelial dry wt. (mg.)	In culture fluid	In mycelial suspension	Total/mg. dry wt.
0	0	4·8	24	67	19
0·1	0	56·6	78	700	14
0·2	0	70·0	114	700	12
0·3	0	72·3	198	760	13
0·4	0	75·9	240	780	13
0·6	0	82·3	198	680	11
1·0	0	83·7	66	320	4·6
3·0	0	93·0	18	140	1·7
0	0·2	36·7	245	400	18
0·1	0·2	87·6	610	1080	19
0·2	0·2	98·9	1030	820	19
0·3	0·2	100·7	830	690	15
0·5	0·2	111·5	670	640	12
1·0	0·2	122·2	180	380	4·6
3·0	0·2	107·8	90	140	2·1

0·1–0·5 μg. biotin/l. Growth was markedly reduced with less than 0·3 μg. biotin/l. so that under these conditions the highest yields of L-oxidase per mg. mycelium were obtained. With 1 μg./l. or higher concentrations of biotin the yields were low at periods of growth between 3 and 20 days. The oxidase itself is not inhibited by biotin even at 10^{-3} M.

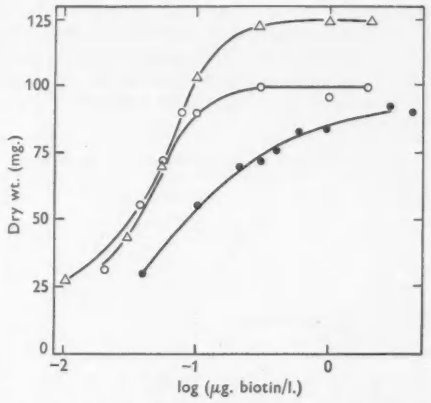

Fig. 2. Effect of biotin on growth of *N. crassa*. Grown for 10 days at 25° on 20 ml. basal medium plus biotin in 50 ml. conical flasks. Strains Beadle 25*a*, △; Cambridge *a*, ○; and CMI 3411, ●.

Strain CMI 3411, which displayed the highest oxidase activity, required more biotin for optimal growth than two other strains, namely Beadle 25*a* and Cambridge *a* (Fig. 2).

Supply of essential nutrients other than biotin. Doubling the amounts of trace elements supplied did not influence the yields of oxidase from strain CMI 3411, but when iron salts were omitted from the medium (1 μg. biotin/l.) the mycelial dry weight was 80 % and yield of oxidase only 15 % of that on the complete medium. With strain CMI 17836, a mutant requiring inositol, on a medium containing 10 μg. inositol/l., growth was 60 % and yield of oxidase 18 % of that obtained with 40 μg. inositol/l.

Source of nitrogen. The addition of casein hydrolysate (0·2 %) to the growth medium containing 0·25 μg. biotin/l. approximately doubled the total yields of the L-enzyme (Tables 3–5) without affecting the growth period or biotin supply required for maximum yields. The casein hydrolysate also increased the growth so that it has little effect on the yield of enzyme per mg. mycelium. The concentration of casein hydrolysate giving maximum yields was 0·2–0·4 % (Table 5); it was equally effective whether hydrolysed by HCl, H_2SO_4 or by HCl in the presence of titanous chloride, as in these experiments. An enzymic digest of casein ('Pronutrin', Herts Pharmaceuticals Ltd., Welwyn Garden City), was not as effective as the casein hydrolysate; the latter contains more of the sparingly soluble amino-acids (cystine and tyrosine) and less biotin than 'Pronutrin'. With casein hydrolysate as the sole source of nitrogen the yield of oxidase was similar to that with both inorganic nitrogen and casein hydrolysate (e.g. Table 5).

The yields of oxidase were low when the sole source of nitrogen was either nitrate or ammonium salts, but were higher on the basal medium which contained both; adding 1 % $(NH_4)_2SO_4$ to the basal medium also reduced the yield. As will be seen from Table 5, some of the changes in the composition of the medium affected the enzymic activity of the culture fluid more markedly than that of the mycelium.

Shaken cultures. Growth was more rapid in the culture flasks of Clayton *et al.* (1944) when shaken

Table 5. *Nitrogen source and yield of* L-*amino-acid oxidase*

(*N. crassa* CMI 3411 grown 9 days on 20 ml. medium (0·25 μg. biotin/l.) in 50 ml. conical flasks. When ammonium tartrate was not present an equimolar amount of sodium potassium tartrate was substituted. Dry weight determined on duplicate culture.)

N source (%)						Oxidase activity (units)			
Ammonium tartrate	NH_4NO_3	$NaNO_3$	$(NH_4)_2SO_4$	Casein hydrolysate	Casein digest ('Pronutrin')	Mycelial dry wt. (mg.)	In culture fluid	In mycelial suspension	Total per mg. dry wt.
0·5	0·1	—	—	—	—	67·8	295	600	13·4
0·5	0·1	—	1·0	—	—	61·6	140	500	8·8
0·5	—	—	0·16	—	—	71·5	38	420	6·4
—	—	0·67	—	—	—	98·6	36	640	6·9
0·5	0·1	—	—	0·1	—	92·0	810	570	14·0
0·5	0·1	—	—	0·2	—	81·7	850	710	19·0
0·5	0·1	—	—	0·4	—	103	1020	960	19·2
0·5	0·1	—	—	0·7	—	118	220	1000	10·3
—	—	—	—	0·7	—	106	420	930	12·7
0·5	0·1	—	—	—	0·1	82·9	730	540	15·3
0·5	0·1	—	—	—	0·4	117	650	540	10·2
0·5	0·1	—	—	—	0·7	130	690	640	10·2

horizontally (120 oscillations/min.) than in stationary flasks, and the mould grew as small fluffy pieces about 5 mm. across. Under all conditions tested (varying growth between 2 and 10 days and varying the composition of the medium with respect to biotin and casein hydrolysate) the yields of oxidase were usually less than 1 unit/mg. mycelium.

Primary reaction catalysed by the oxidase

For each molecule of L-alanine oxidized by the culture fluid, one molecule of pyruvic acid was formed and half a molecule of O_2 consumed (e.g. 11·3 μmol. L-alanine added, 10·6 μmol. pyruvic acid formed and 5·45 μmol. O_2 used). As the culture fluid contained a relatively high concentration of ammonium salts, the production of ammonia could not be accurately measured. The ammonium salts were removed without losing oxidase activity by dialysis against running tap water for 14 hr. One molecule NH_3 was formed from each molecule of L-alanine oxidized. The overall reaction is therefore

$$R.CHNH_2.COOH + \tfrac{1}{2}O_2 = R.CO.COOH + NH_3. \quad (1)$$

By analogy with other amino-acid oxidases it may be expected that hydrogen peroxide is formed in the primary reaction; this would be decomposed by the catalase present in the culture fluid. In fact hydroxylamine or hydrogen cyanide, which inhibit the catalase, increased the rate of O_2 uptake, and the final quantity of O_2 consumed rose to one molecule per molecule of L-amino-acid. With preparations of oxidase free from catalase (see below), one molecule of O_2 was consumed per molecule of amino-acid; addition of catalase halved the amount of O_2 consumed. It may therefore be taken that hydrogen peroxide is formed in the primary reaction. It should therefore be possible to couple the oxidation of ethanol with that of amino-acids in the presence of relatively high concentrations of catalase (cf. Keilin & Hartree, 1945). The culture fluid contained insufficient catalase for this reaction, but with the addition of red blood cells the O_2 consumption was increased to 0·90 molecule O_2 per molecule L-phenylalanine when ethanol was added.

Factors affecting the activity of the oxidase

Preparation of oxidase for kinetic studies. Strain CMI 3411 was grown for 12 days at 25° in 1 cm. depth of basal medium with the addition of 0·25 μg. biotin/l. Culture flasks were those of Clayton *et al.* (1944) originally designed for the production of penicillin. The culture fluid at the end of the growth contained both L-amino-acid oxidase and catalase. The fall in activity on storage in the refrigerator with octanol as a preservative, was up to 50 % in a week but often less. The culture fluid contained about 11 mg. non-dialysable N/l.; no precipitate occurred on adding trichloroacetic acid. A stable dry powder was obtained after concentrating the culture fluid to one-fifth the original volume at reduced pressure (bath temperature 40–50°). A precipitate appeared and was removed on the centrifuge. The clear golden-brown supernatant was cooled to 0°, 1·7 vol. acetone cooled to −5° was added with stirring and the suspension centrifuged. The precipitate was dried *in vacuo* in the centrifuge tubes over H_2SO_4 and solid NaOH. The precipitate was ground to a powder and stored *in vacuo* over H_2SO_4 and solid NaOH. It contained catalase activity and 50–60 % of the oxidase activity of the culture fluid; no activity was lost after storing for 6 months at room temperature.

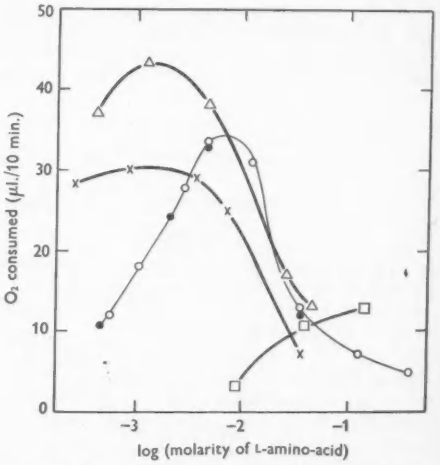

Fig. 3. Substrate concentration curve. For procedure and calculation see text. L-alanine, ○; DL-alanine, ●; L-glutamate, □; DL-leucine, △; L-phenylalanine, ×.

For use the powder was dissolved in water; a small amount of insoluble residue was removed by centrifuging. In most preparations 1 mg. was equivalent to about 6 units of oxidase. The material consisted of the soluble constituents of the medium and contained less than 1 % protein. This preparation is a convenient form of the enzyme for use as an analytical reagent (e.g. Gibson & Wiseman, 1951).

Concentration of substrate. The relation between activity and concentration of substrate was studied for alanine, glutamic acid, leucine and phenylalanine at 30° and pH 8·2 with O_2 as the gas phase (Fig. 3). The main compartments of the manometer vessels contained 1·5 ml. 0·1 M-pyrophosphate buffer and the amino-acid in a total volume of 4 ml. The oxidase (5 mg. acetone-precipitated material in 0·5 ml. water) was added from the side arm when thermal equilibrium was established. To avoid

complications arising from the lag period in the first few minutes after mixing, the activities were obtained from the O_2 consumed in the period beginning 5 min. after mixing; the 5–25 min. period was used for concentrations higher than 0·02 M, the 5–10 min. period for the lowest concentrations of phenylalanine and of leucine, and the 5–15 min. period for other values. The substrate concentrations plotted were the mean concentrations during the period used for the measurement of the O_2 uptakes. These were calculated from the initial substrate concentrations and the O_2 consumed, on the basis of equation (1).

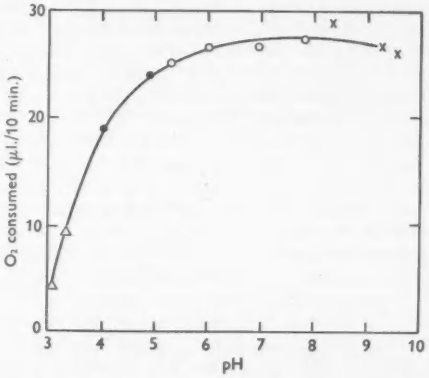

Fig. 4. pH-Activity curve. Manometer flasks contained: 1·5 ml. 0·1 M-buffer as below, 1·87 mg. L-phenylalanine and 2·5 ml. water. After thermal equilibration 0·5 ml. oxidase solution (5 mg. acetone-precipitated material) was added from side arm. Buffers: lactate, △; acetate, ●; phosphate, ○; pyrophosphate, ×. Temp., 30°; gas, O_2.

It will be seen that the enzyme is inhibited by high (above 0·01 M) concentrations of substrate, except in the case of glutamic acid. For alanine and leucine, the plot of activity against the logarithm of substrate concentration is bell-shaped as with certain other enzymes, e.g. L-amino-acid oxidase of snake venom (Zeller & Maritz, 1945) and cholinesterase (Alles & Hawes, 1940). The points obtained with DL-alanine lie on the same curve as those for L-alanine. With phenylalanine, the activity is almost independent of its concentration between 5×10^{-4} M and 3×10^{-3} M—and this amino-acid is therefore specially suitable for determining the activity of oxidase preparations. A theory of inhibition by high concentrations of substrate has been given by Haldane (1930).

pH. The pH activity curve shows a broad maximum between pH 6 and 9·5. At pH 4 the activity is approximately 70 % of the maximum (Fig. 4).

Hydrogen acceptors. Ferricyanide and reducible dyes can replace O_2 as a hydrogen acceptor. The rate of O_2 consumption at pH 8·2 in 0·033 M-pyrophosphate was 4·5 times the rate of anaerobic reduction of ferricyanide measured manometrically at pH 7·4 in 0·025 M-$NaHCO_3$ by the method of Quastel & Wheatley (1938). 2:6-Dichlorophenol indophenol (0·075 mg.) was decolorized in 3·3 min. when incubated anaerobically at 30° and pH 8·2 with 90 units of oxidase and 1·67 mg. DL-phenylalanine in 2 ml. 0·033 M-pyrophosphate; in the absence of substrate the dye was decolorized in 50 min. The rate of reaction with O_2 was therefore about twenty times the rate with 2:6-dichlorophenol indophenol. Methylene blue was reduced much more slowly; the reduction of 0·075 mg. required 3 hr. in the presence of phenylalanine; no reduction occurring without added substrate.

The rate of oxidation in O_2 (1 atm.) was 3·4 times that in air.

Temperature. The optimum was about 45° when the activity was determined at pH 8·2 as already described (Table 6). Between 20 and 40° the rate of reaction was approximately doubled for every 10° rise in temperature. Heat stability is optimal between pH 6 and 7 and the substrate protects the enzyme against heat inactivation. After heating at 55° and pH 6 for 5 min. 55 % of the activity was lost; only 13 % was lost in the presence of 5×10^{-3} M-L-leucine.

Table 6. *Effect of temperature on oxidase activity*

(Manometer flasks contained: 1 ml. 0·1 M-pyrophosphate buffer pH 8·2, 7·5 mg. acetone-precipitated oxidase and water to 4 ml. DL-phenylalanine (3·75 mg. dissolved in 0·5 ml. pyrophosphate buffer) was added from the side arm after 10 min. equilibration. Gas: O_2.)

Temp. (°)	O_2 consumed (µl./10 min.)
20	10
30	20
35	28
40	40
45	51
50	39

Inhibitors. Of the substances tested on the acetone-precipitated material, the following did not affect either the rate of O_2 consumption or the final quantity of O_2 consumed: 2:6-dichlorophenol indophenol (1 mM final concentration); D-phenylalanine (6 mM); iodoacetic acid, *p*-chloroacetophenone, 8-hydroxyquinoline, 2:4-dinitrophenol, DL-lactic acid, kojic acid, benzoic acid and phthalic acid (10 mM); DL-*N*-methylleucine (20 mM), $(NH_4)_2SO_4$ (50 mM); sodium fluoride (100 mM) and octanol (saturated solution). Crystal violet, and methylene blue reduced the rate of reaction while 0·01 M-HCN, azide or hydroxylamine increased the

O_2 consumption by inhibiting the catalase. The effects of these five substances and also atebrine, quinine and $CuSO_4$ were examined with a purified preparation of oxidase free from catalase (see below); the oxidase was not affected by 0·01 M-HCN, hydroxylamine, or azide nor by 10^{-3} M-quinine sulphate, but 0·01 M-atebrine inhibited 15%, 10^{-3} M-crystal violet inhibited 69% and 2×10^{-3} M-methylene blue inhibited about 30%. In the absence of any buffer the inhibition by $CuSO_4$ was 63% at 10^{-2} M and 15% at 10^{-3} M. In the presence of 0·033 M-pyrophosphate buffer there was no inhibition by 10^{-2} M-$CuSO_4$.

Prosthetic group

Purification of oxidase. For bulk preparation the concentration of sucrose in the culture was reduced to 1·3% and that of ammonium tartrate to 0·2%; 0·2% NH_4Cl was added. This did not reduce the yield of oxidase. Strain CMI 3411 was grown at 25° in 1 cm. depth of 6·5 l. medium (0·25 μg. biotin/l.). After 12 days the culture fluid (5·6 l.) contained 150,000 units of oxidase (2400/mg. non-dialysable N). Preliminary experiments showed that the enzyme was adsorbed by cupric hydroxide, but not by alumina Cγ or calcium phosphate. To the culture fluid were added 214 ml. 10% $CuSO_4.5H_2O$ and then 74 ml. 2 N-NaOH with continuous stirring, and the precipitate of $Cu(OH)_2$ was centrifuged off. The amounts of copper present in the supernatant or precipitate did not affect the measurement of enzyme activity. Most of the activity remained in the supernatant, but was adsorbed by the precipitate formed on the addition of a further 118 ml. 10% $CuSO_4$. The precipitate of $Cu(OH)_2$ was centrifuged off and suspended in 100 ml. 0·1 M-acetate buffer pH 6 (110,000 units of oxidase). After dialysing the suspension overnight against running water, most of the oxidase was still adsorbed to the insoluble $Cu(OH)_2$. This was centrifuged off and resuspended in 120 ml. water, and 2 N-NH_4OH (15 ml.) was added slowly with continuous stirring until the $Cu(OH)_2$ was dissolved (66,000 units). This solution was dialysed overnight against running tap water (49,000 units) and centrifuged. The blue supernatant (39,000 units) was saturated with $(NH_4)_2SO_4$ by dissolving 175 g. in the 230 ml. solution. No precipitate was obtained at 80% saturation, but the material which was precipitated on complete saturation contained 32,000 units of oxidase. It was suspended in 20 ml. water, dialysed overnight and centrifuged. The clear, pale-yellow supernatant contained 25,000 units and 2·5 mg. non-dialysable N. To remove the last traces of Cu the enzyme was re-precipitated by saturation with $(NH_4)_2SO_4$. The precipitate (16,000 units) was collected by centrifuging, suspended in 15 ml. water, dialysed against running tap water for 15 hr. and centrifuged. The 24 ml. clear, pale-yellow supernatant contained 12,000 units of oxidase and 1·1 mg. non-dialysable N; it was free from catalase. The activity was slowly lost (30% in 2 weeks) on storing at $-10°$.

An alternative method of removing the Cu from the enzyme is to dissolve the $Cu(OH)_2$ fraction in 0·2 M-pyrophosphate buffer pH 8. Most of the Cu can then be removed by dialysis. This procedure was sometimes, but not always, accompanied by large losses of the enzyme activity.

Presence of FAD in the purified oxidase. The enzyme preparation was examined for riboflavin, riboflavin phosphate and FAD by descending paper chromatography. Whatman no. 1 papers were washed with 0·1 N-HCl, then with distilled water and dried before use. After developing the chromatograms they were dried at 80° in the dark; 0·03 μg. of each flavin could be located by its fluorescence in ultraviolet light. The R_F values of riboflavin, riboflavin phosphate and FAD were 0·8, 0·14 and 0·05 respectively, with a solvent mixture consisting of *n*-butanol (40 vol.)–acetic acid (10 vol.)–water (50 vol.) and 0·8, 0·19 and 0·08 with a solvent mixture of *tert.*-butanol (60 vol.)–pyridine (25 vol.)–water (15 vol.). To concentrate the flavins in the oxidase preparation they were extracted by phenol as follows: saturated $(NH_4)_2SO_4$ solution (4 ml.) and 40% trichloroacetic acid (0·2 ml.) were added to 4 ml. of the purified oxidase solution and the mixture twice extracted by 1 ml. portions of phenol liquefied by adding water. The combined phenol extracts were shaken with 0·4 ml. water and 4 ml. $CHCl_3$ and centrifuged. About 0·2 ml. of the upper (aqueous) layer was then transferred to another tube and neutralized. When 30 μl. samples of this solution were examined chromatographically a single fluorescent spot (yellow-green) was observed; it had the same R_F values as FAD in both systems. Neither riboflavin nor riboflavin phosphate was detected.

The oxidase preparation showed a green fluorescence which was equivalent to $0·08 \times 10^{-6}$ M-riboflavin; it was increased to that of $0·53 \times 10^{-6}$ M-riboflavin by heating at 100° for 3 min. or by acidifying (one-tenth vol. N-HCl) and neutralizing to pH 7, suggesting that most of the FAD in the oxidase preparations was combined to protein, in a non-fluorescent form.

The fluorescence of riboflavin or riboflavin phosphate is 7·1 times that of FAD at pH 7 (Burch, Bessey & Lowry, 1948; cf. Singer & Kearney, 1950*a*). A solution of FAD was hydrolysed by heating at pH 0·6 (HCl) and 100° for 30 min.; after cooling and neutralizing the fluorescence was increased 8·1 times (from 0·13 to 1·05 times that of riboflavin). Similar treatment of the enzyme preparation increased the fluorescence to the equivalent of $4·4 \times 10^{-6}$ M-riboflavin, i.e. 8·3 times that after

only brief exposure to acid. This behaviour confirmed the identification of the oxidase flavin as FAD.

FAD can be identified and estimated using the protein component of kidney D-amino-acid oxidase (Warburg & Christian, 1938). The *Neurospora* L-amino-acid oxidase activated the kidney D-oxidase protein when the *Neurospora* enzyme had been heated at 100° for 3 min. or acidified (one-tenth vol. N-HCl) and neutralized. The untreated *Neurospora* enzyme did not activate the kidney D-amino-acid oxidase. The D-oxidase was not affected by the *Neurospora* enzyme in the presence of excess (2×10^{-8} g.mol.) FAD. As will be seen from Table 7, about 4×10^{-6} M-FAD was liberated by heating or acidifying the preparation of *Neurospora* L-amino-acid oxidase.

Table 7. *Replacement of FAD in the* D-*amino-acid oxidase system by the* L-*amino-acid oxidase of* Neurospora

(Manometer flasks contained: 1 ml. 0·2 M-pyrophosphate buffer, pH 8·2, 2 mg. D-alanine, red blood cells (0·01 ml. blood) as a source of catalase, and FAD or *Neurospora* L-oxidase preparation in a total volume of 3·3 ml. After equilibration, 0·2 ml. D-oxidase apo-enzyme was added from the side arm. Temp., 40°; gas, O_2.)

Addition		O_2 consumed in 5–15 min. after mixing (μl.)	Free FAD in *Neurospora* oxidase (μM/l.)
FAD (g.mol. $\times 10^{-9}$)	*Neurospora* oxidase (ml.)		
—	—	14	—
1·42	—	51	—
2·13	—	63	—
2·84	—	70	—
—	1·0 (untreated)	12	0
—	0·50 (heated)	58	3·6
—	0·48 acidified and neutralized)	64	4·5

Effect of alanine and $Na_2S_2O_4$ *on the absorption spectrum of the oxidase preparation.* The purified oxidase (3 ml.) was mixed with 0·3 ml. 0·2 M-pyrophosphate buffer pH 7·5, and the absorption spectrum (*A*) was determined between 370 and 550 mμ. (Fig. 5). The solution was transferred to a Thunberg tube which was evacuated and 6 mg. solid DL-alanine was added from the stopper. After 10 min. the tube was filled with nitrogen and the solution transferred to the absorption cell: a stream of nitrogen was passed over the surface of the liquid until the absorption spectrum (*B*) had been measured. Curve *C* was obtained after 2 mg. $Na_2S_2O_4$ had been dissolved in the solution.

It will be seen from Fig. 5 that the DL-alanine decreased the light absorption of the oxidase preparation; when the spectrum (*B*) in the presence of alanine was subtracted from the original (*A*), curve *D* was obtained with maxima at 385 and 470 mμ., and a minimum at about 415 mμ. This shows that some of the FAD in the oxidase can be reduced by the alanine. $Na_2S_2O_4$ further reduced the light absorption. The total decrease ($A - C$) in the extinction coefficient at 450 mμ. ($\Delta \log I_0/I_{(450\ m\mu.)}$) was 0·042. Assuming that this change was due

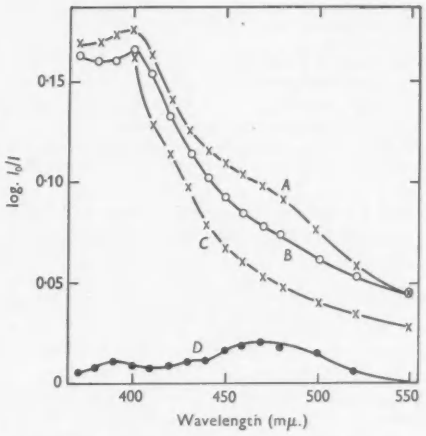

Fig. 5. Effect of alanine and $Na_2S_2O_4$ on absorption spectrum of L-aminoacid oxidase. For procedure see text. Curve *A*: oxidase preparation; curve *B*: oxidase + DL-alanine; curve *C*: oxidase + DL-alanine + $Na_2S_2O_4$; curve *D*: *A*–*B*.

entirely to the reduction of FAD and that the corresponding change in the molecular extinction coefficient ($\Delta\epsilon_{450\ m\mu.}$) is $-1{\cdot}05 \times 10^4$, the oxidase contained $\dfrac{0{\cdot}042 \times 3{\cdot}3}{1{\cdot}05 \times 10^4 \times 3{\cdot}0} = 4{\cdot}4 \times 10^{-6}$ M-FAD. The value for $\Delta\epsilon_{450\ m\mu.}$ was obtained by measuring $\Delta \log I_0/I_{450\ m\mu.}$, when 2 mg. $Na_2S_2O_4$ was added to 3 ml. 10^{-5} M-FAD or riboflavin. When $Na_2S_2O_4$ was added to the acid-inactivated oxidase $\Delta \log I_0/I_{450\ m\mu.}$ corresponded to $4{\cdot}3 \times 10^{-6}$ M-FAD in the original oxidase preparation.

The values for the FAD content of the oxidase obtained by spectrophotometry agree with those obtained with the protein component of the D-amino-acid oxidase (4×10^{-6} M) and by fluorimetry ($4{\cdot}4 \times 10^{-6}$ M).

Specificity

The relative rates of oxidation of the twenty amino-acids tested were similar both in the purified oxidase and in crude mycelial extracts. In addition to the thirty-three amino-acids previously found to be oxidized (Bender & Krebs, 1950), the enzyme attacks canavanine (private communication from Dr N. H. Horowitz), arginine, citrulline, glycine and

Table 8. *Rates of oxidation of amino-acids**

(Manometer flasks contained: 3 ml. culture fluid from strain CMI 3411, 1·0 ml. 0·1 M-pyrophosphate buffer, pH 8·3. Amino acid ($2·25 \times 10^{-5}$ g.mol.) weighed into side bulb and moistened with 0·5 ml. buffer. Temp., 30°; gas, O_2. The figures in the last column are comparable with the q_{O_2} values of Bender & Krebs, 1950.)

Amino-acid	O_2 absorbed (μl.) after 10 min.	20 min.	30 min.	90 min.	120 min.	Maximum rate (μl. O_2/10 min.)	Relative rate
L-Phenylalanine	66	148	195	260	261	82	(52)
L-Arginine	12	34	68	227	228	34	22
L-Canavanine	44	101	176	260	264	75	47
L-Citrulline	31	88	158	265	265	70	45
α:ε-Diaminopimelic acid (natural compound)	60	134	213	252	254	79	50
Glycine	2	5	7	15	22	25	1·5

* Certain q_{O_2} values for the *Neurospora* L-oxidase given in Table 3 of the paper by Bender & Krebs were incorrectly calculated from the amounts of O_2 consumed. The corrected q_{O_2} values are phenylalanine: 52, tyrosine: 34, *p*-amino-phenylalanine: 32, β-6-methoxyquinolyl(4)alanine: 63, β-pyridyl(4)alanine: 60, and histidine: 75.

natural α:ε-diaminopimelic acid (Table 8). This last amino-acid probably has the *meso* configuration (Work, 1951).

Some observations on the formation of D-*amino-acid oxidase by* Neurospora

In previous experiments when the growth period was 14 days (Bender *et al.* 1949), the D-enzyme was found in some strains (e.g. Beadle 25*a*) but not in others (e.g. CMI 3411 and Cambridge *a*). Further experiments showed that the enzyme was formed by all strains when tested after 7 days' growth. The highest activities were obtained with strains Beadle 25*a* and Chilton *a*. The production of the D-oxidase by these strains was not affected by varying the concentration of biotin in the culture medium from 0·2 to 5 μg./l. With Chilton *a* grown on the basal medium plus biotin (5 μg./l.) the amount of D-oxidase per mg. dry weight found in mycelial suspensions was nearly doubled by the addition of 0·2 % casein hydrolysate to the culture medium; no enzyme was found when 1 % $(NH_4)_2SO_4$ had been added. The highest Q_{O_2} obtained in the suspensions (1) was much less than that obtained with L-phenylalanine and mycelial suspensions of CMI 3411 (115).

Distribution of L-*oxidase in other mould species.* Knight (1948) has described an L-amino-acid oxidase in the *Penicillia* and in *Aspergillus niger*. This enzyme differs from the *Neurospora* enzyme in its specificity and sensitivity to inhibitors. Knight examined *N. crassa*, but did not find an L-amino-acid oxidase, probably because his growth conditions (shaken cultures in a corn-steep liquor (2 %)-lactose (2 %) medium) were unfavourable for oxidase production. The oxidase is not destroyed by the acetone-drying procedure used by Knight.

Several mould species were grown under conditions which favour the production of the L-oxidase by *Neurospora* (14 days' stationary culture in the basal medium plus 0·01 % yeast and 0·3 % malt extracts). L-Phenylalanine was readily oxidized by the mycelial extracts from *Melanospora destruens* and the culture fluid contained 1·2 units of oxidase activity per ml. The oxidase was not detected in the other species; L-phenylalanine or L-alanine did not affect the small endogenous respiration of mycelial extracts from *Chaetonium caprinum*, *Fusarium lini*, *Gibberella zeae*, *Nectria cinnabarina*, *Penicillium chrysogenum* Q 176, or *Trichoderma viride*. With *Aspergillus niger* CMI 594 and *Sordaria fimicola*, these amino-acids depressed the endogenous respiration.

DISCUSSION

Factors influencing the yield of L-*oxidase.* The most remarkable factor controlling the yield is the amount of biotin in the culture medium; the yield of enzyme per mg. mycelium is highest when growth is limited by the supply of biotin and is considerably reduced by more than 1 μg. biotin/l. In the absence of detailed information on the mechanism of action of biotin on growth generally it is hardly possible to offer an explanation of the observed effect.

Some data suggest a connexion between slow growth and high L-oxidase activity. The two strains which produce the most oxidase (CMI 3411 and Cambridge *a*) grow slower and sporulate less readily than other strains, especially Beadle 25*a* and Emerson *a*, which produce little oxidase. The oxidase activity is low when growth is stimulated by shaking the culture or by providing an optimal supply of biotin. However, this relation between growth and oxidase activity is not general; limitation of growth by the supply of iron to strain CMI 3411 or of inositol to the mutant, strain CMI 17836 decreases the oxidase activity per unit of weight of mycelium.

The yields of L-oxidase per mg. mycelium are similar with an inorganic source of nitrogen or with a completely organic source (casein hydrolysate).

This is in contrast to the findings with the L- and D-oxidases of *Penicillium* (Knight, 1948; Emerson, Puziss & Knight, 1950) and the D-oxidase of *Neurospora* where the yields of enzyme are considerably lower with an inorganic source of nitrogen than with an amino-acid source.

Prosthetic group. The purified oxidase contained 1 g.mol. bound FAD per 11,000 g. non-dialysable nitrogen, whilst riboflavin and riboflavin phosphate were not detectable. All preparations of the oxidase absorbed considerably more light below 480 mμ. than was expected from the FAD content. The change in absorption spectrum due to the reduction of FAD was therefore difficult to measure, but, on the addition of a substrate, a definite effect was found and this may be taken as strong evidence for the view that FAD is the prosthetic group of the oxidase.

FAD is also the prosthetic group of the L-amino-acid oxidase of snake venom (Singer & Kearney, 1950*a*), of kidney D-amino-acid oxidase (Warburg & Christian, 1938) and of glycine oxidase (Ratner, Nocito & Green, 1944). The prosthetic group of the kidney L-amino-acid oxidase described by Blanchard, Green, Nocito & Ratner (1945) is riboflavin phosphate.

Turnover number. The purified enzyme oxidized 2100 mol. phenylalanine/mol. FAD/min. at 30°. This is of the same order as the turnover numbers of the L-amino-acid oxidase of snake venom (Singer & Kearney, 1950*b*) and the mammalian D-amino-acid oxidase (Warburg & Christian, 1938), namely 3100 and 1440 respectively.

Comparison with some other oxidases. The more striking properties of the *Neurospora* oxidase include the ability to oxidize a wide range of L-α-amino-acids, the broad pH optimum and the inhibition by high substrate concentrations. The L-amino-acid oxidase of snake venom is also inhibited by high concentrations of substrate (Zeller & Maritz, 1945). The *Neurospora* oxidase is not affected by many substances such as benzoic acid, 2:4-dinitrophenol, iodoacetic acid or octanol which inhibit either the kidney D-amino-acid oxidase (Klein & Kamin, 1941), the amino-acid oxidases of *Penicillium* (Knight, 1948; Emerson *et al.* 1950) or the L-amino-acid oxidase of snake venom (Zeller & Maritz, 1945).

SUMMARY

1. Seventeen strains of *Neurospora crassa* and three of *N. sitophila* were examined for amino-acid oxidases. Under suitable conditions, which are described, both an L-α-amino-acid and a D-α-amino-acid oxidase were detected in all strains. The activity of both enzymes varied considerably from strain to strain. An L-oxidase was also found in *Melanospora destruens*; it was absent from several other species.

2. The highest yields of L-amino-acid oxidase were obtained with *Neurospora crassa* CMI 3411 in stationary culture on a liquid medium containing sucrose, ammonium tartrate, 0·2% casein hydrolysate, inorganic salts, and 0·1–0·5 μg. biotin/l. The oxidase activity in the mycelium and also in the culture fluid was highest after 12 days' growth at 25°.

3. Concentrations of biotin above 1 μg./l. considerably reduce the yield of L-amino-acid oxidase.

4. The kinetics of the L-amino-acid oxidase were studied. The pH-activity curve has a broad maximum between pH 6 and 9·5. High concentrations of substrate inhibit.

5. The specificity was not changed by 4·5-fold purification of the oxidase from the culture fluid.

6. Bound flavin-adenine dinucleotide (FAD) was present in the purified oxidase (1 g. mol./11,000 g. non-dialysable nitrogen) as shown by the activation of the D-amino-acid oxidase apo-enzyme, fluorimetry and paper chromatography. Riboflavin phosphate and riboflavin were not detected. Anaerobic addition of alanine decreased the optical density of the enzyme preparation between 380 and 500 mμ.; this change was that which is characteristic of the chemical reduction of flavo-proteins. It may therefore be taken that FAD is the prosthetic group of the L-amino-acid oxidase.

7. The turnover number was estimated to be 2100 molecules phenylalanine per minute at 30°.

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Studies in Carotenogenesis

2. CAROTENE PRODUCTION BY *PHYCOMYCES BLAKESLEEANUS*: THE EFFECT OF DIFFERENT AMINO-ACIDS WHEN USED IN MEDIA CONTAINING LOW CONCENTRATIONS OF GLUCOSE

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In a previous investigation (Garton, Goodwin & Lijinsky, 1951) it was shown that, using a medium containing 3% (w/v) glucose, carotene synthesis in *Phycomyces blakesleeanus* was independent of the amino-acid used as the nitrogen source with the exception that glycine enhanced carotene production. Amongst the amino-acids which had no effect was L-valine, from which a hypothetical five-carbon building unit such as β-methylcrotonaldehyde (Karrer, Helfenstein & Widmer, 1928) could conceivably arise and promote carotene synthesis. The only other naturally occurring amino-acid containing a terminal dimethyl grouping is L-leucine. Such a grouping would appear to be essential in an active repeating unit acting as a carotene precursor, but in media containing 3% (w/v) glucose L-leucine was as ineffective as L-valine.

It was possible, in media containing high concentrations of glucose, for stimulation of carotene synthesis by L-leucine or L-valine to be masked; it was therefore decided to investigate the action of these amino-acids in media containing sufficient glucose to produce a reasonable mycelial growth, but insufficient to produce more than small amounts of carotene. The observations of Garton *et al.* (1951) indicated that the most useful glucose concentration would be 1% (w/v); this produces about 75% of the maximal mycelial growth, whilst the carotene production is less than 10% of the maximal.

EXPERIMENTAL

The general cultural and analytical procedures employed were described in detail in the first paper of this series (Garton *et al.* 1951). The various media were made by altering the glucose concentration of the standard medium, generally to 1% (w/v), and varying the N source according to requirements; the salt and aneurin concentrations were unaltered. All experiments were carried out under natural illumination.

Replacement cultures. Well-developed mycelia which could be easily handled and transferred to fresh media were obtained by culturing the fungus on filter paper (Whatman no. 50) in Petri dishes (11 cm. diameter). Small glass beads (2–3 mm. diameter) were added in sufficient numbers to cover the bottom of the dishes and 45 ml. of the medium added to each dish; this is just sufficient to cover the beads. The filter papers were then placed in position on the beads and the dishes sterilized. Inoculation was carried out by pipetting the spore suspension on to the filter paper. When the mycelia were at the appropriate stage of growth they were transferred to a new medium in another Petri dish by lifting out the filter paper and adhering mycelium, washing it with sterile water in an inoculating cabinet and placing it carefully on the new medium. Glass beads are not essential in the replacement media, but they are necessary in the first cultures in order to obtain reproducible mycelial growths. Glass beads are much better for supporting the filter paper than sand, which had previously been suggested for this purpose by Raaf (1941). Small amounts of sand inevitably get into the mycelia and, as they are impossible to remove completely, dry weight determinations cannot be carried out. After growth is completed, the filter paper can be removed by peeling it away from the fungal mat, which can then be examined in the usual way.

RESULTS

In the first experiment three media were used; all contained 1% (w/v) of glucose, whilst the N sources were either L-leucine, L-asparagine or L-valine (*c*, 0·35, 0·2 and 0·676% (all w/v) respectively). It

was intended that the nitrogen content of the media should remain constant at 0·0374 % (w/v); this was possible with the first two amino-acids, but the growth rate on the equivalent amount of L-valine was found to be so small that the nitrogen concentration had to be doubled in order to get a reasonable growth rate. The results obtained on these media (Table 1) show that the growth rate is very much less on L-leucine and L-valine than on L-asparagine, 10–14 days being required to reach maximal mycelial dry weight compared with 4 days. The final growth is also somewhat reduced. Carotene synthesis, on the other hand, is considerably increased both relatively (from 0·03 to 0·07–0·09 % dry weight) and absolutely (from 18 to 32–41 μg.); in young cultures lipid production is more rapid than general mycelial growth, but the final amount produced is not significantly different from that formed on L-asparagine.

to provide a final carbon concentration of 0·464 % (w/v).

The results of a typical series of experiments with these media are recorded in Table 2. Dry weight and lipid production are approximately the same in all media, whilst more carotene is produced on media containing L-leucine or L-valine than on any of the others; under these conditions glycine has no stimulatory action on carotene production either in the presence or absence of L-leucine or L-valine. When the concentrations of carotene are considered, the stimulation by L-leucine and L-valine is very obvious (Fig. 1), the values in the presence of these amino-acids being two to four times greater than those obtained on the other media. It will also be noted that the effect persists throughout the growth period examined (4–14 days) and that L-leucine is considerably more effective than L-valine.

Table 1. *Dry weight, lipid and carotene production by* Phycomyces *on media containing* 1 % (*w/v*) *glucose and either* 0·2 % (*w/v*) (= 0·0374 % N) L-*asparagine*, 0·35 % (*w/v*) (= 0·0374 % N) L-*leucine, and* 0·624 % (*w/v*) (= 0·0748 % N) L-*valine*

(The amounts are those produced in a standard culture flask (8 oz. medicine bottle containing 15 ml. of medium).)

Period of growth (days)	Amino-acid in medium: L-Asparagine Dry wt. (mg.)	L-Asparagine Lipid (mg.)	L-Asparagine Carotene (μg.)	L-Valine Dry wt. (mg.)	L-Valine Lipid (mg.)	L-Valine Carotene (μg.)	L-Leucine Dry wt. (mg.)	L-Leucine Lipid (mg.)	L-Leucine Carotene (μg.)
4	66	15·8	14	—	—	—	21	7·9	5
5	65	15·9	11	—	—	—	23	7·3	7
7	60	10·0	18	16	5·3	6	36	12·5	19
10	52	5·4	12	37	11·5	23	46	14·2	41
14	46	4·1	10	50	9·0	32	46	13·8	34

It was then decided to find out (*a*) if L-leucine and L-valine stimulated carotenogenesis when the fungus was growing at its normal rate, and (*b*) if glycine, which stimulates synthesis in glucose-rich media, is also effective in media low in glucose, and (*c*) if mixtures of glycine and either L-leucine or L-valine were more effective than the corresponding mixtures with L-asparagine. Media containing the following amino-acid constituents were used: (*a*) L-asparagine, (*b*) glycine, (*c*) L-asparagine + glycine, (*d*) L-leucine + L-asparagine, (*e*) L-leucine + glycine, (*f*) L-valine + L-asparagine and (*g*) L-valine + glycine. All the media contained 0·0374 % (w/v) of nitrogen; in those containing two amino-acids one-half of the nitrogen was provided by each component. As changes in the carbon content of the medium in the concentration range used in this experiment can affect the amount of carotene synthesized (Garton *et al.* 1951), the concentration of carbon in all these media was kept at 0·464 % (w/v); this was achieved by adding the amino-acids at levels equivalent to a nitrogen concentration of 0·0374 % (w/v) and then adding sufficient glucose

The stimulation recorded in the experiments just described appears to be the maximal effect obtainable with L-leucine and L-valine, because it was found that doubling the concentrations of these amino-acids had no further effect on carotene production.

In order to find out if the effect of L-leucine and L-valine was observed in cultures which were growing only very slightly, as well as in actively growing mycelia, a series of experiments was carried out using replacement cultures. The fungus was cultured on the standard medium (2·5 % (w/v) glucose and 0·2 % (w/v) L-asparagine) on filter paper supported by glass beads, in Petri dishes; when the mycelia were 4–5 days old, i.e. when almost fully grown but containing very little carotene, they were transferred to fresh media. In the first series of transference experiments the media used contained 1 % (w/v) glucose and 0·2 % (w/v) of either L-valine, L-leucine or L-asparagine. The mean base-line levels of dry weight, lipid and carotene were obtained by analysing a number of 4- to 5-day-old mats inoculated the same time as, and cultured alongside, the mats which were transferred. The results are re-

Table 2. *Dry weight, lipid and carotene production by* Phycomyces *on media containing glucose and amino-acids*

(The concentrations of the amino-acids were so arranged that the N content of all the media was 0·0374 % (w/v) and in those media containing two amino-acids each provided one-half of the N. The total C content of all the media was kept constant by adding sufficient glucose to provide a final C content of 0·464 % (w/v). The amounts are those produced in a standard culture flask (8 oz. medicine bottle containing 15 ml. of medium).)

Period of growth (days)	Amino-acid in medium																					
	L-Asparagine			Glycine			L-Asparagine + glycine			L-Leucine + L-asparagine			L-Leucine + glycine			L-Valine + L-asparagine			L-Valine + glycine		
	Dry wt. (mg.)	Lipid (mg.)	Carotene (μg.)	Dry wt. (mg.)	Lipid (mg.)	Carotene (μg.)	Dry wt. (mg.)	Lipid (mg.)	Carotene (μg.)	Dry wt. (mg.)	Lipid (mg.)	Carotene (μg.)	Dry wt. (mg.)	Lipid (mg.)	Carotene (μg.)	Dry wt. (mg.)	Lipid (mg.)	Carotene (μg.)	Dry wt. (mg.)	Lipid (mg.)	Carotene (μg.)
4	68	19	9	43	6	12	67	10	11	58	11	24	42	8	16	50	12	13	30	5	10
6	54	10	9	60	10	6	43	12	8	53	7	23	56	10	24	48	9	16	53	12	13
8	54	9	10	52	9	9	54	9	7	47	5	21	51	7	24	43	7	17	44	8	14
10–11	51	6	6	46	6	8	43	4	8	44	3	21	49	5	22	41	5	15	43	7	13
13–14	48	5	7	41	2	5	46	4	6	44	3	14	43	6	13	37	3	10	37	4	10

corded in Table 3 and show that whilst the total dry weight of the transferred cultures has increased 3 days after transference on all the media it was greatest on the medium containing L-asparagine; the carotene production, however, was very much greater on the media containing the other amino-acids, whilst the amount of lipid produced had not appreciably altered in any culture. The dry weight

Fig. 1. The concentration of carotene produced by *Phycomyces* at various stages of growth when grown on media containing 0·0374 % (w/v) of N and 0·464 % (w/v) of C. The N applied as different amino-acids either singly or as binary mixtures, the C (other than that in the amino-acids) as glucose. ●—●, L-Asparagine; ×—×, glycine; ▲—▲, L-asparagine + glycine; ■—■, L-leucine + L-asparagine; ○—○, L-leucine + glycine; △—△, L-valine + L-asparagine; □—□, L-valine + glycine.

had dropped somewhat on all the media 5-6 days after transference, mainly owing to the disappearance of lipid from the mycelia; the carotene content had also dropped a little in the media containing L-asparagine and L-valine, but continued to increase on the L-leucine medium. The very great stimulatory effect of L-leucine and L-valine in these conditions can be appreciated by reference to Fig. 2, in which the concentrations of carotene in the three mycelia are compared. This figure also emphasizes again the superiority of L-leucine over L-valine in this respect.

In the final experiment, well formed mycelial mats were transferred to media containing no glucose but

Table 3. *Dry weight, carotene and lipid production by well developed mycelia*

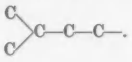
(Mycelia, 4 days old; grown on a medium containing 2·5% (w/v) of glucose, transferred to media containing 1% (w/v) of glucose and 0·2% (w/v) of either L-valine, L-leucine or L-asparagine. The amounts are those produced in an 11 cm. Petri dish containing 45 ml. of medium.)

	Amino-acid in medium								
	L-Asparagine			L-Valine			L-Leucine		
Time after transference (days)	Total dry wt. (mg.)	Lipid (mg.)	Carotene (μg.)	Total dry wt. (mg.)	Lipid (mg.)	Carotene (μg.)	Total dry wt. (mg.)	Lipid (mg.)	Carotene (μg.)
0	273	77	56	273	77	56	273	77	56
3	401	76	163	374	97	273	350	81	364
5	370	53	135	347	70	229	319	48	441

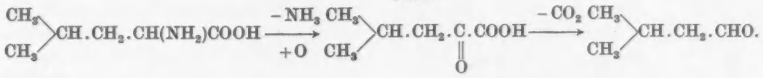
only the amino-acids L-asparagine, L-leucine, L-valine or glycine as the sole nitrogen and carbon sources. Table 4 shows that under these conditions, the media containing 0·2% L-leucine or L-valine synthesized a little carotene whilst those containing L-asparagine or glycine synthesized none. Synthesis took place to a slight but definite extent on all the media when the amino-acid concentration was raised to 1% (w/v); no differences between the amino-acids could, however, be observed. The increases noted are very small compared with those obtained when small amounts of glucose are present (Table 3). In all cases the dry weight of the mycelia dropped after transference mainly owing to the rapid disappearance of the lipid fractions.

DISCUSSION

The demonstration that L-valine can, in the absence of excess glucose, stimulate carotenogenesis in *Phycomyces* strongly suggests that the repeating unit in the synthesis contains the C_5 skeleton:



L-Leucine is even more active than L-valine and may furnish the C_5 residue by losing its carboxyl carbon; in this way a decarboxylation following oxidative deamination would yield *iso*valeraldehyde, which could function as the repeating unit, thus:



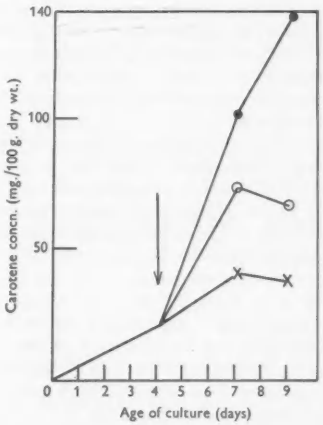


Fig. 2. The concentration of carotene produced by fully grown mycelial mats of *Phycomyces* transferred to media containing 1% (w/v) glucose and either 0·2% (w/v) L-valine, L-leucine or L-asparagine. The arrow indicates the day the mycelia were transferred. ●—●, L-Leucine; ×—×, L-asparagine; ○—○, L-valine.

This possibility is supported by the observation of Ehrlich (see Foster, 1949) that L-leucine is the specific precursor of the *iso*valeraldehyde found in the fusel oil produced during yeast fermentation. That L-valine is less active than L-leucine may be because *iso*valeraldehyde is more easily formed from L-leucine than from L-valine. Whether this compound is, in fact, the active repeating unit and, if so, whether it is dehydrogenated before or after condensation is under investigation. It is of considerable importance to settle this because Bonner & Arreguin (1949), studying the biogenesis of rubber, an analogous problem, concluded that the unsaturated compound β-methylcrotonic acid was the active repeating unit; whilst implicit in the ideas of Porter & Lincoln (1950) on carotenoid synthesis in tomatoes is the assumption that the repeating unit is a saturated compound. They suggest that carotenoids are synthesized in tomatoes by the stepwise removal of four hydrogen atoms from a saturated C_{40} precursor.

As *Phycomyces* grows at almost the same rate on a medium containing NH_4^+ as the sole nitrogen source as it does on a medium containing L-asparagine or glycine, it must be concluded that the very

Table 4. *Dry weight, carotene and lipid production by well developed mycelia*

(Mycelial mats (4–5 days old) of *Phycomyces* transferred to media containing no glucose but one of the four amino-acids, L-leucine, L-valine, L-asparagine, and glycine, at a concentration of 0·2 or 1·0 %, w/v.)

Time after transference (days)	Amino-acids in media																	
	At 0·2 % (w/v) level								At 1 % level									
	L-Valine			L-Leucine			L-Asparagine			Glycine			L-Valine			L-Leucine			L-Asparagine		
	Dry wt. (mg.)	Lipid (mg.)	Carotene (μg.)	Dry wt. (mg.)	Lipid (mg.)	Carotene (μg.)	Dry wt. (mg.)	Lipid (mg.)	Carotene (μg.)	Dry wt. (mg.)	Lipid (mg.)	Carotene (μg.)	Dry wt. (mg.)	Lipid (mg.)	Carotene (μg.)	Dry wt. (mg.)	Lipid (mg.)	Carotene (μg.)	Dry wt. (mg.)	Lipid (mg.)	Carotene (μg.)
0	206	48	46	206	48	46	206	48	46	206	48	46	273	77	56	273	77	56	273	77	56
3	187	33	56	199	24	53	191	29	39	177	28	33	244	42	76	241	43	93	245	51	87
6	170	18	53	133	11	44	149	8	44	154	16	45	231	36	88	220	28	86	221	32	72

slow growth on L-leucine or L-valine is due to the slow rate of deamination of these amino-acids; for on them growth does eventually reach a level almost equal to that on other amino-acids (Garton *et al.* 1951). The rate of glucose catabolism by *Phycomyces* developing on a medium containing only L-leucine or L-valine as the nitrogen source probably provides a sufficient concentration of active intermediates to fix, as cellular material, all the NH_3 arising from the slow deamination of the amino-acids. The carbon residues from these amino-acids (probably α-keto-*iso*valeric and -*iso*caproic acids) would thus be utilized for cellular growth only to a minor extent and be mostly available for, *inter alia*, carotene synthesis. In the case of rapidly deaminated amino-acids (e.g. L-asparagine) the carbon residue would much more likely be mainly utilized for general growth purposes. In the case of media containing a mixture of L-leucine or L-valine and L-asparagine or glycine, growth is normal because of the presence of the latter two amino-acids, but carotene synthesis is above normal because of the presence of L-leucine and L-valine.

It is important to realize that if L-leucine and L-valine had supported a normal growth rate of *Phycomyces*, the fact that they can provide an active intermediate for carotene synthesis might have gone unsuspected, for a rapid provision of ammonia would have probably resulted in the utilization of most of the keto acids for synthesis of cellular material. When well formed mycelia are transferred to fresh media containing 1 % (w/v) of glucose and 0·2 % (w/v) of an amino-acid as the nitrogen source, much less growth occurs as measured by nitrogen assimilation (Goodwin & Willmer, 1951) than in newly germinated mycelia. Under these conditions if a deaminated amino-acid were a specific intermediate in carotene synthesis some increase in pigment production should be obtained whatever the rate of deamination of that amino-acid. The observation that L-leucine and L-valine are still active in replacement media whilst the other amino-acids remain inactive, emphasizes the specificity of these two amino-acids in stimulating carotenogenesis; if L-asparagine or glycine had any specific stimulatory effect one would have expected it to show up in replacement cultures. These replacement experiments also show that carotenogenesis can be separated completely from lipogenesis, for the lipid content of the transferred mycelia always decreased although carotene production was proceeding rapidly.

The disappearance of the stimulatory action of L-leucine and L-valine when the culture media contain high concentrations of glucose (Garton *et al.* 1951) indicates that the specific unit provided by these amino-acids is readily formed during the dissimilation of the excess glucose.

The failure of L-leucine and L-valine to form appreciable amounts of carotene in replacement cultures in the absence of glucose is taken to indicate that energy is required for one or both of two reactions: (*a*) to transfer the amino-acids into the fungal cells, as is the case with certain bacteria (Gale, 1951); (*b*) to effect the condensation or the dehydrogenation, or both, of the repeating units; glucose dissimilation appears to be necessary in order to provide this energy.

The role of glycine in the biogenesis of carotenoids remains obscure. It is the only amino-acid which stimulates synthesis in the presence of high concentrations of glucose (Garton *et al.* 1951), but no such effect is noted in the presence of only 1 % (w/v) of glucose.

Lipid production. Although not primarily concerned with lipogenesis this investigation has brought out two important points which must be briefly considered. In the first place *Phycomyces* cultured on media containing only 1 % (w/v) of glucose contain, as soon as they are fully grown, a normal concentration of lipid (20–30 g./100 g. dry weight), but this is quickly lost as the mycelia age; on the higher glucose concentrations the disappearance of the lipid is much slower (Garton *et al.* 1951). The utilization of the mycelial lipid also occurs when well formed (4-day-old) mats are transferred to media containing only 1 % (w/v) of glucose. Secondly, when the culture medium contains 1 % (w/v) of glucose and either L-leucine or L-valine as the sole nitrogen source, the total mycelial growth rate is much more reduced than is the rate of lipid synthesis, so that in young cultures the concentration of lipid is much greater than normal (about twice). This rate is relatively not as great as the rate on media supporting normal rate of total growth; further, the amount of lipid synthesized on L-leucine and L-valine is never more than in other media, so that when maximal growth is attained the lipid concentration is not significantly greater than normal. This further demonstrates that the stimulatory effect of L-leucine and L-valine is confined to carotene; they stimulate the relative rate of lipogenesis but have no effect on the amount synthesized.

SUMMARY

1. The effect on the carotene production by *Phycomyces blakesleeanus* of using various amino-acids as the nitrogen source in media containing 1 % (w/v) glucose has been studied.

2. L-Valine and L-leucine whether used alone or in conjunction with glycine or L-asparagine stimulate carotenogenesis, in some cases as much as fourfold. Glycine and L-asparagine either alone or together have no such effect.

3. Transference of well formed mycelial mats to media containing 1 % (w/v) glucose and various amino-acid combinations also results in considerably increased carotene production only on those media containing either L-leucine or L-valine.

4. The relative rate of lipogenesis is increased in young mycelia growing on media containing 1 % (w/v) glucose and either L-leucine or L-valine. The total amount of lipid synthesized is, however, not different from that produced on media containing other amino-acids.

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Observations on the Oxidation of Homogentisic Acid in Urine

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Homogentisic acid, which is considered to be an intermediate in the metabolism of tyrosine and phenylalanine, is excreted in a number of different conditions. The most widely studied of these, which is caused by a genetic inability to oxidize homogentisic acid, was termed alcaptonuria, owing to the characteristic darkening of the urine on standing. It has also been shown (Sealock & Silberstein, 1940; Rogers & Gardner, 1949) that, normally, ascorbic acid plays some part in the metabolism of homogentisic acid, since the latter has been claimed to be present in the urine of scorbutic subjects and of premature infants (Levine, 1949). In both these latter cases, but not in congenital alcaptonuria, the administration of ascorbic acid stops the excretion of homogentisic acid. Dietary amino-acid imbalance may also interfere with the normal metabolism of tyrosine and phenylalanine and result in excretion of homogentisic acid (Glynn, Himsworth & Neuberger, 1945; Neuberger & Webster, 1947). Homogentisic acid is the reduced form of 1:4-benzoquinone-2-acetic acid, both together constituting an oxidation-reduction system. The high positive redox potential of benzoquinone-acetic acid, however, makes it appear extremely unlikely that it could exist for any appreciable length of time in a biological medium. It is therefore surprising that Fishberg (1948) reported its presence in urines from a case of enterogenous cyanosis, from scorbutic animals and from cases of rheumatic fever. The last observation was of special interest since it suggested a possible relationship of rheumatic fever to a vitamin C disturbance, a relationship for which some evidence already existed (Rinehart, 1938). Since the methods for detecting the presence of benzoquinoneacetic acid depend largely on its oxidizing properties, special care must be taken to avoid the development of oxidizing substances in urine after its collection. This could arise, for instance, as a result of the growth of nitrite-forming bacteria. The nitrite so produced would not only give several of the tests used by Fishberg (1948) to demonstrate benzoquinoneacetic acid, but might oxidize any homogentisic acid present to the quinone and thus account for her unexpected findings. It was therefore decided to investigate the action of oxidants on homogentisic acid in urine; and secondly, in the light of these results, to re-investigate Fishberg's claims to have found benzoquinoneacetic acid in urines from rheumatic fever cases.

EXPERIMENTAL

Paper chromatography was used to demonstrate the presence of homogentisic acid in urines. The property of ready oxidation to the quinone has been used to provide a delicate and specific test. Homogentisic acid was separated from interfering substances in urine on paper chromatograms, and estimated quantitatively, after elution, by treatment with phosphomolybdate and measurement of ultraviolet absorption at 355 mμ. (Consden & Stanier, 1951).

Reactions of homogentisic acid in urine towards oxidizing conditions were followed by means of ultraviolet absorption spectrophotometry. Homogentisic acid and benzoquinoneacetic acid have characteristic absorption spectra, sufficiently different from each other to enable one to be detected in the presence of the other.

Materials. Homogentisic acid was a pure sample kindly supplied by Dr A. Neuberger, F.R.S. 1:4-Benzoquinone-2-acetic acid was prepared from this by oxidation with chromic acid (Mörner, 1912). It liberated 96% of the theoretical quantity of I_2 from acidified KI, and by titration with alkali had an equivalent of 162 (theoretical, 166).

Paper chromatography. In *n*-butanol-acetic acid-water (4:1:5 by vol.), homogentisic acid had an R_F value of 0·80. A satisfactory reagent was ammoniacal $AgNO_3$, which produced an immediate black or brown spot on the chromatogram; 1 μg. could be clearly detected in this way. In this solvent system, benzoquinoneacetic acid was almost entirely converted to homogentisic acid. On chromatograms developed with *n*-butanol in an atmosphere of NH_3 oxidation of homogentisic acid quickly occurred, producing a brown spot at the origin and the presence of the quinone in this spot could be demonstrated by spraying with acidified KI-starch solution (1% KI and 1% starch made acid with a few drops 6N-HCl) which produced a slowly developing blue colour (R_F, 0). Owing to the instability of the quinone at high pH, the KI reaction gives much less colour on chromatograms developed for long periods such as 17 hr. than when developed after only 2–4 hr. Benzoquinoneacetic behaved exactly as homogentisic acid in *n*-butanol-NH_3 (1 μg. being detectable), but, in the acetic acid system, was converted to homogentisic acid, since no brown spot positive to KI was present, but instead, a spot with R_F of

0·80 was demonstrated with ammoniacal $AgNO_3$. A more specific test for homogentisic acid is carried out as follows: the solution to be tested is applied to Whatman no. 4 paper and duplicate chromatograms developed in *n*-butanol-acetic acid for 2–4 hr. One chromatogram, after drying, is sprayed with the silver reagent. The other is spotted on a suitable area with bromothymol blue indicator and then sprayed with the acidified KI-starch reagent. It is then hung for 1·5–2 min. in a chamber containing NH_3 vapour, produced by a 3% NH_3 solution; during this time the indicator spot becomes blue. The chromatogram is then removed and after a short airing becomes acid, as shown by the change of colour of the indicator spot to yellow. A blue spot now appears in the same position as that occupied by the Ag spot on the control chromatogram. The procedure can be repeated, which usually enhances the blue KI spot. 1 μg. homogentisic acid in 5 μl. urine can readily be detected in this way.

Quantitative estimation of homogentisic acid. Homogentisic acid (25–50 μg.) was run on paper chromatograms for 4–6 hr. in butanol-acetic acid. The areas containing the homogentisic acid, as determined by a 'marker' chromatogram treated with ammoniacal $AgNO_3$, were cut out and eluted with water in the apparatus described by Consden, Gordon & Martin (1947), but instead of running the eluate into a capillary tube, the liquid was allowed to flow down a thin strip of filter paper into a 5 ml. graduated flask. An end of the strip was touched against the corner of the cut when it was sufficiently wet, and the strip then remained in position by capillary attraction. After about 0·1 ml. of eluate had been collected, KH_2PO_4 (0·4 ml. of 1% solution) and ammonium molybdate (0·4 ml. of 5% solution in 5N-H_2SO_4) were added and the solution made to volume with water. After allowing to stand for 1 hr. the optical densities at 355 mμ. were read. A portion of the chromatogram not containing homogentisic acid was treated in the same way to provide the control solution. This paper blank had a negligible absorption when measured against the reagents alone. Comparison of the absorptions with the standard (Consden & Stanier, 1951) gave 90–100% recoveries. Several other methods were tried for the estimation of small quantities of homogentisic acid from paper chromatograms. Estimation of the optical densities of eluates at 290 mμ. was not successful owing to comparatively high and variable paper blanks, which could not be eliminated. The KI titration method of Neuberger (1947) was too insensitive for the small amounts handled. The method of Park & Johnston (1949) for the estimation of reducing sugars, consisting of production of Prussian blue from ferricyanide, was applied to homogentisic acid, and although it is even more sensitive than the phosphomolybdate method when dealing with 2–10 μg., it could not be applied to homogentisic acid from chromatograms as the paper blanks were high and variable.

Recovery from urine. 150 μl. urine containing 30 μg. homogentisic acid were applied to paper, with intermediate drying, across a line 4–5 cm. in length. The development of the chromatogram, the elution of the homogentisic acid and treatment with phosphomolybdate were carried out as described above. The blank was obtained from a similar area of the chromatogram, which was free from reducing substances as shown by the Ag 'marker'. The densities at 355 mμ. measured against the blank gave recoveries of 90%. When larger amounts of homogentisic acid in urine were estimated in this way, better recoveries were obtained, e.g. 100% recovery for 60 μg.

Ultraviolet absorption spectra

Solutions were measured in a Unicam spectrophotometer, Model SP 500, using 1 cm. quartz cells. For measurement, homogentisic acid or benzoquinoneacetic acid (0·004% in water) was mixed with an equal volume of M/15 phosphate buffer, pH 6·8. M/30 Phosphate was the control solution. Solutions of homogentisic acid, if kept at 0°, showed no alteration even after several months, whereas benzoquinoneacetic acid required to be freshly prepared before measurement, since marked changes in the absorption curve are liable to occur on standing, similar to those shown in Fig. 3*C*.

Action of nitrous acid on homogentisic acid. To homogentisic acid (0·1 ml. of 0·1%) was added freshly prepared 1% $NaNO_2$ solution (0·1 ml.); M/15 phosphate buffer (2 ml.) was added, followed by 0·1N-HCl (0·5 ml.). The solution (acid to methyl orange) was allowed to stand for 5 min. and was then made up to 5 ml. with M/15 phosphate (final pH, 6·7). A control was prepared containing all the constituents except homogentisic acid. The absorption curve is shown in Fig. 2*B*. No change in the absorption curve was observed without acidification of the nitrite-containing solution.

Action of NH_3 *on homogentisic acid.* 0·1N-NH_3 (1 ml.) was added to a solution consisting of 1% homogentisic acid (0·2 ml.) and M/15 phosphate buffer (1 ml.). The solution darkened and liberated I_2 from acidified KI solution. After allowing to stand for 10–15 min., the solution was made to 10 ml. with phosphate buffer. The absorption curve measured against M/15 phosphate buffer is shown in Fig. 3*A*. The absorption curve of this solution measured after 17 hr. is also shown in Fig. 3*C*.

Effect of ascorbic acid on homogentisic acid treated with nitrous acid, or with ammonia. After treatment as described above with HNO_2 or NH_3, ascorbic acid (0·15 mg.) was added to the final buffered solution, and the absorption then measured (Fig. 2*C*).

Effect of HNO_2 *on homogentisic acid treated with* NH_3. N-NH_3 was added to a solution consisting of 1% homogentisic acid (0·5 ml.) and M/15 phosphate (2 ml.) until just alkaline to thymol blue. The solution was made up to 5 ml. with water and allowed to stand for 15 min. To 2 ml. was added $NaNO_2$ (1 ml. 0·1%) and N-HCl (0·2 ml.) until acid to methyl orange. After 15 min. the solution was diluted to 10 ml. with M/15 phosphate. The absorption curve measured against a control solution containing all the above constituents except homogentisic acid is shown in Fig. 3*B*.

Spectrophotometry of urine containing added homogentisic acid. The absorption curve of urine from a healthy individual is shown in Fig. 4*A*. The urine was diluted 200 times with water and phosphate buffer (final concentration M/30), and the absorption was measured against M/30 phosphate buffer. The same urine containing 0·4% added homogentisic acid and diluted similarly gave the curve shown in Fig. 4*B*. To demonstrate the effects of HNO_2 and NH_3, the urines containing homogentisic acid were treated with these reagents as described above in the case of homogentisic acid solutions and absorption spectra after dilution were measured against the homogentisate-free urine treated with the same reagents. Similar experiments were carried out on urines containing 0·02% added homogentisic acid, but for spectrophotometry the final dilutions were 40 instead of 200 times.

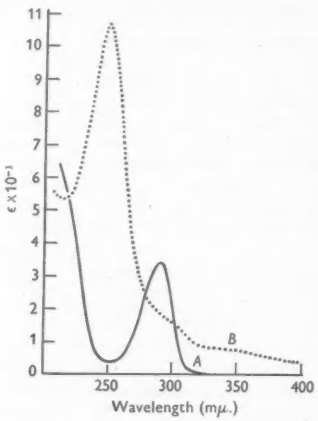

Fig. 1.

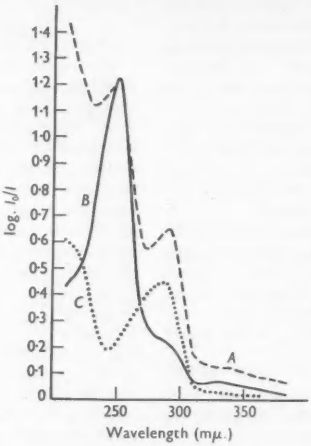

Fig. 2.

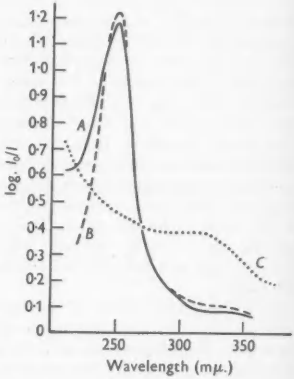

Fig. 3.

Fig. 1. Ultraviolet absorption spectra of homogentisic acid (*A*) and benzoquinoneacetic acid (*B*) in M/30 phosphate buffer, pH 6·8. (All spectra shown in Figs. 1–6 were derived from solutions made up with M/30 phosphate buffer pH, 6·6–6·8.)

Fig. 2. Ultraviolet absorption spectra of a mixture of homogentisic acid and benzoquinoneacetic acid (*A*), homogentisic acid after treatment with nitrous acid (*B*) and homogentisic acid treated with nitrous acid or ammonia, then re-treated with ascorbic acid (*C*). Final concentrations of all solutions correspond to 0·002% homogentisic acid or benzoquinoneacetic acid.

Fig. 3. Ultraviolet absorption spectra of homogentisic acid after treatment with ammonia (*A*), treatment of this solution with nitrous acid (*B*), and solution treated with ammonia measured after 17 hr. (*C*). Final concentrations of all solutions correspond to 0·002% homogentisic acid.

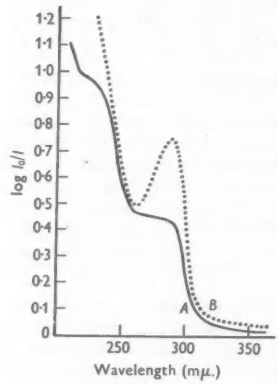

Fig. 4.

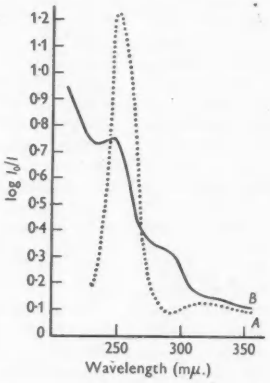

Fig. 5.

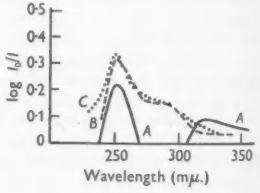

Fig. 6.

Fig. 4. Ultraviolet absorption spectra of normal urine (*A*) the same urine containing 0·4% added homogentisic acid (*B*). Urines diluted 200 times.

Fig. 5. Ultraviolet absorption spectra of urine containing homogentisic acid (0·4%) after treatment with nitrous acid (*A*), and ammonia (*B*). Both solutions were diluted 200 times, and absorptions were measured against the homogentisate-free urine treated and diluted similarly.

Fig. 6. Ultraviolet absorption spectra of urine containing homogentisic acid (0·02%) after treatment with nitrous acid (*A* and *B*) and ammonia (*C*). Control solutions for curves *B* and *C* were homogentisate-free urines treated similarly and for curve *A* was untreated homogentisate-urine. Final dilution of urines, 40 times.

RESULTS AND DISCUSSION

The absorption curves of homogentisic acid ($\epsilon = 3430$ at $\lambda_{max.}$ 290 mμ.) and benzoquinoneacetic acid ($\epsilon = 10790$ at $\lambda_{max.}$ 250 mμ.) are shown in Figs. 1*A* and *B*, respectively. The absorption curve for a mixture of these two substances is shown in Fig. 2*A*. If these curves are compared with those which Fishberg (1948) claimed to be the curves of benzoquinoneacetic acid, it will be seen that the latter correspond much more closely, as regards their maxima and minima, with homogentisic acid.

Treatment of homogentisic acid with nitrous acid or ammonia results in almost complete conversion to the quinone (Figs. 2*B* and 3*A*). This reaction is largely reversed by excess of ascorbic acid (Fig. 2*C*). With ammonia, presumably atmospheric oxidation takes place. Retreatment of the ammonia-treated homogentisic acid with nitrous acid appears to remove substances absorbing at short wavelengths (Fig. 3*B*). Homogentisic acid in urine can be easily detected spectophotometrically, if present in sufficiently large amounts (Fig. 4*B*). Treatment of such urine with nitrous acid or ammonia results in the formation of benzoquinoneacetic acid, though, as would be expected, owing to the presence of reducing substances, the conversion may not be so complete as with homogentisic acid alone. This is illustrated in Fig. 5*B*, where atmospheric oxidation in the presence of ammonia gives a curve similar to that of a mixture of homogentisic acid and benzoquinoneacetic acid (Fig. 2*A*). On the other hand, nitrous acid results in more complete oxidation (Fig. 5*A*) in spite of the competing urinary substances, e.g. urea. This effect of nitrous acid could also be reproduced in urine containing nitrite-forming bacteria. Urine containing 0·2% homogentisic acid was inoculated with nitrite-producing bacteria obtained from a contaminated sample of urine which gave reactions positive for nitrite. After incubation, the inoculated urine, on acidification, gave the absorption curve for benzoquinoneacetic acid.

The effects of nitrous acid and ammonia could be demonstrated on urines containing much smaller amounts of homogentisic acid, owing to the smaller dilutions which had to be employed in spite of the interfering absorption of other urinary constituents which is relatively high. Urine containing 0·02% homogentisic acid, when treated with nitrous acid, gave the curve shown in Fig. 6*A* when measured against the untreated homogentisic acid containing urine, and the curve shown in Fig. 6*B* when measured against the homogentisate-free urine treated with nitrous acid. A similar curve (Fig. 6*C*) was obtained after treatment with ammonia and measuring against the homogentisate-free urine also treated with ammonia.

Solutions of homogentisic acid, when made alkaline with ammonia and allowed to stand for a short time, liberated iodine from acidified potassium iodide solution. This reaction was observed even in urine containing only 0·02% homogentisic acid. The presence of homogentisic acid in urine may therefore be demonstrated by three tests described above: (*a*) by paper chromatography; (*b*) by the development of oxidizing properties on treatment with ammonia; and (*c*) by the disappearance of the peak at 290 mμ. in the ultraviolet absorption curve after treatment with ammonia or nitrous acid, and the corresponding appearance of a peak or inflexion at 250 mμ.

Examination of rheumatic fever urines

Urines from febrile rheumatic fever children gave absorption spectra similar to urine containing added homogentisic acid (Fig. 4*B*). However, this substance was absent as shown by paper chromatography. Treatment of the urine with ammonia failed to modify the absorption curve, and it is concluded that the peak at 290 mμ. is due to excess of substance other than homogentisic acid. This is probably uric acid, since the intensity of this spot on the paper chromatogram was greater than that of a spot obtained under similar conditions from normal urine, and preliminary experiments showed that the absorption curve of uric acid was unaffected after treatment with ammonia. Some of the rheumatic fever urines after standing for 24 hr. liberated iodine from potassium iodide, and gave positive tests with most of the reagents used by Fishberg (1948) for demonstrating benzoquinoneacetic acid. However, these tests are not specific, as they are also given by nitrite. Nitrites were demonstrated in these urines by the red colour produced after addition of sulphanilic acid, acidification and addition of alkaline β-naphthol. This reaction is negative with homogentisic acid and benzoquinoneacetic acid.

Examination of scorbutic guinea pig urines

Urines from scorbutic guinea pigs showed no evidence in their ultraviolet absorption curves of any excess of substance having a maximum at 290 mμ. This was found also for ethyl acetate extracts of the acidified urines. The absence of homogentisic acid was confirmed by paper chromatography.

Examination of urines from rats with methaemoglobinaemia

Methaemoglobinaemia was produced in a group of rats by feeding nitrite. The urines were pooled, acidified, extracted with ethyl acetate, and the concentrated extracts were chromatographed on paper in both butanol-acetic and butanol-ammonia. With

ammoniacal silver nitrate no spot corresponding to homogentisic acid was observed in the butanol-acetic acid chromatogram. In butanol-ammonia, at least six reducing spots were present, with R_F values of 0·1–0·8. A duplicate chromatogram was sprayed with alkaline diazotized sulphanilic acid. Ten spots appeared of various shades of yellow and pink. Six of these appeared to be in positions identical with those of the six reducing spots. Another duplicate chromatogram was sprayed with methyl orange to demonstrate acidic substances. The upper five spots positive to the sulphanilic acid reagent appeared to be acidic. In the above three groups of urines, since 20 μl. failed to give a positive paper chromatographic test, it is concluded that homogentisic acid was present, if at all, in a concentration of less than 0·005 %.

From the results obtained, it is apparent that great precautions are necessary before the presence of benzoquinoneacetic acid in the urine can be assumed to indicate that this substance is actually being excreted. Bacterial contamination of the urine readily leads to production of ammonia, and somewhat less frequently to nitrite formation. Both of these conditions (the latter, after acidification) will cause almost immediate oxidation of homogentisic acid to benzoquinoneacetic acid. It is therefore only permissible to conclude that any benzoquinoneacetic acid detected was excreted in this form, if bacterial contamination of the urine has been strictly excluded. Moreover, since several of the tests used for benzoquinoneacetic acid depend upon its power as an oxidizing agent, nitrites may themselves be responsible for these reactions in the absence of either benzoquinoneacetic acid or homogentisic acid.

There is no indication in the paper of Fishberg (1948) that the necessary safeguards were taken against bacterial contamination. Our own failure to obtain any evidence of benzoquinoneacetic acid excretion by patients with rheumatic fever strongly suggests that the positive results of Fishberg should be attributed to nitrite formation in the urine after its passage. The isolation of benzoquinoneacetic acid from her case of autotoxic enterogenous cyanosis appears to rest upon much stronger evidence. But here again it would appear more probable that what was actually excreted was homogentisic acid and that its subsequent oxidation was due to either urinary ammonia, or to nitrite. The presence of nitrite in the blood and urine of patients with autotoxic enterogenous cyanosis is well authenticated (Evans, Enzer, Eder & Finch, 1950), and it is therefore possible that, in such cases, benzoquinoneacetic acid would be formed on acidification of the urine, even in the absence of bacterial contamination, provided of course that homogentisic acid were present. This would imply that the patient was suffering from both methaemoglobinaemia and alcaptonuria.

SUMMARY

1. Paper chromatography of homogentisic acid and benzoquinoneacetic acid is described, and a method is given for the determination of homogentisic acid in urine, after separation on paper chromatograms.

2. The ultraviolet absorption spectra of these two substances have been determined.

3. The behaviour of homogentisic acid in urines under oxidizing conditions has been followed by means of ultraviolet spectrophotometry. Conversion to the quinone takes place in urine, if nitrous acid is present or when the urine is made alkaline.

4. By paper chromatography and by spectrophotometry, homogentisic acid and benzoquinoneacetic acid were found to be absent from the urines of rheumatic fever cases, scorbutic guinea pigs and rats with experimental methaemoglobinaemia.

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A Study of the Cytochrome System and some other Aspects of the Respiration of *Aerobacter aerogenes*

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The object of this work was the study of the interrelationship between the components of cytochrome in *Aerobacter aerogenes*, the effect of metal deficiency on the development of cytochrome in relation to the metabolism of these micro-organisms, and the effect of azide both on their cytochrome system and their respiratory activities.

Whereas in aerobic cells with a typical cytochrome system comprising the components a_3, a, b and c, much knowledge is now available on the properties of and the relationship between these components, very little is known as to the properties and functions of the components in an atypical cytochrome system.

A. aerogenes shows an atypical cytochrome system comprising the components a_2, a_1 and b_1 and is thus suitable for the study of these problems. In this bacterium, the component a_2 is much more highly developed than in any known micro-organism, and is therefore easier to study.

Moreover, the fact that *Aerobacter* can be cultivated in a metal-deficient medium, as was shown by Waring & Werkman (1944), makes it possible to follow the effect of metal deficiency on the development and synthesis of the different components of cytochrome in relation to the respiratory activity.

Finally, since the introduction of azide as an inhibitor of respiration and of the activity of certain catalytic systems, an extensive literature on this subject has revealed that its inhibitory effect is more specific than that of cyanide. However, it was shown recently by Aubel & Szulmajster (1947) that in *Escherichia coli* azide does not affect the rate of oxygen uptake in presence of glucose. *A. aerogenes* has also been found suitable for further analysis of this phenomenon.

MATERIAL AND METHODS

Methods of cultivation. A. aerogenes, strain I, from the M.R.C. Unit for Chemical Microbiology, Cambridge, was used in the course of this work. It was cultivated either in a nutrient broth with glucose or in a special metal-deficient medium. The nutrient broth had the following composition: peptone, 10 g.; NaCl, 5 g.; 'Lemco' meat extract, 5 g.; glucose, 10 g.; and distilled water 1 l. It was sterilized by autoclaving. Incubation was carried out at 30° in plugged Erlenmeyer flasks, each fitted with a capillary tube reaching to the bottom of the flask. Air was bubbled through the medium by means of a small mechanical pump, and cotton wool plugs in glass tubes acted as air filters. After 15–18 hr. incubation, the bacteria were harvested, washed twice with distilled water, and suspended in 0·1 M-phosphate buffer, pH 6·8.

Metal-deficient medium was prepared according to Waring & Werkman (1944), with the modifications described below. For the preparation of 1 l. of medium, 4 g. Na_2HPO_4, 1 g. KH_2PO_4 and 1 g. $(NH_4)_2SO_4$ were dissolved in about 200 ml. redistilled water in a separating funnel, shaken with about 15 mg. 8-hydroxyquinoline in 1 ml. $CHCl_3$ for 30 sec., left for 1 or 2 min. and extracted with several lots of 10 ml. $CHCl_3$, until the $CHCl_3$ layer was no longer coloured by the metal complex. This treatment was repeated six times, the medium being finally extracted with six lots of $CHCl_3$ to remove all traces of the chelating agent. $MgSO_4.7H_2O$ (0·1 g.) was added, the solution was boiled to remove $CHCl_3$, made up to 1 l. in an Erlenmeyer flask, covered with a well fitting beaker and autoclaved. A 20% glucose solution was treated separately in the same way, autoclaved, and 50 ml. were added to 1 l. of the sterile medium. It was found necessary to sterilize the glucose separately, otherwise a brown insoluble pigment was produced which subsequently interfered with spectroscopic examination.

The inoculations were made in the following way: 250 ml. Erlenmeyer flasks, containing 100 ml. metal-deficient medium, were inoculated from a normal culture. After 24 hr. growth, 0·2 ml. of this was used to inoculate 1 l. of medium. The cultures were incubated at 30°, air being bubbled through them as before. The growth after 24 hr. was about one-third of that obtained from a purified medium to which iron had been added.

Spectroscopic methods. 0·5 ml. of a thick bacterial suspension in 0·1 M-phosphate buffer pH 6·8 was placed in a small flat-bottomed glass tube giving a depth of 3–5 mm. This bacterial suspension was then examined by means of a Zeiss microspectroscope ocular fitted to a microscope, as described by Keilin & Hartree (1946). Aeration of the bacterial suspension was carried out by means of a fine glass capillary fitted to an air pump and frothing was prevented by a very small amount of octyl alcohol (octan-2-ol, 'caprylic alcohol') (about 0·001 ml./0·5 ml. of bacterial suspension).

To reinforce the absorption bands, the suspension of micro-organisms was mixed with an equal volume of glycerol and cooled in liquid air, as described by Keilin & Hartree (1949).

Manometric methods. After an initial dry-weight determination, an aqueous suspension of bacteria was diluted with 0·1 M-phosphate buffer pH 6·8, to give a dry weight of 10 mg./ml. The experiments were carried out in Barcroft differential manometers at 30°. The bacterial suspension

(1–3 mg. dry weight) and the substrate were added in the right-hand flask to a sufficient amount of 0·1 M-phosphate buffer to give a final volume of 3·0 ml. 20% (w/v) KOH (0·3 ml.) was placed in the centre well. The left-hand flask contained 3·3 ml. distilled water.

Aerobic CO_2 output and respiratory quotients were measured by the method of Dixon & Keilin (1933). The special manometric flasks contained a total liquid volume of 3·6 ml.

Determination of dehydrogenase activity. The experiments were carried out at 30° in Thunberg tubes. Each tube contained 1 ml. $5{\cdot}2 \times 10^{-4}$ M-methylene blue, 0·3 ml. 0·1 M-substrate, and enough 0·1 M-phosphate buffer to give a total volume of 3 ml., with 0·3 ml. bacterial suspension in the hollow stopper. The tubes were evacuated and twice washed with O_2-free N_2.

Estimation of glucose, pyruvic acid and lactic acid. In the early experiments, glucose was estimated by two methods: the colorimetric method of Folin & Malmros (1929) as described by Umbreit, Burris & Stauffer (1945), which gives the total reducing value, and the method of Keilin & Hartree (1948) utilizing a specific glucose oxidase (notatin). As the two methods gave similar results, the simpler colorimetric method was used in later experiments. Pyruvic acid was estimated by the method of Friedmann & Hangen (1943), using toluene as the organic solvent, and lactic acid by the method of Barker & Summerson (1941), as described by Umbreit *et al.* (1945).

RESULTS

Cytochrome system in bacteria grown in metal-rich medium (nutrient broth)

Washed bacteria have a negligible respiration, and show either very feeble or no absorption bands, the cytochromes being in the oxidized state. The absorption band of component a_2 in the oxidized state is sometimes visible at 645 mμ. On the addition of a suitable substrate (5% glucose was used in the following experiments) the cytochrome is quickly reduced, and the characteristic absorption bands appear. *A. aerogenes* now shows three absorption bands in the visible region: band b_1 at 560 mμ., a_1 at 590 mμ. and a_2 at 630 mμ. Of these, the band b_1 is the strongest. It is a rather wide, asymmetric band, with a reinforcement on the side towards the shorter wavelengths. The a_1 band is very feeble, while the a_2 band is sharp and almost as strong as b_1.

When the bacterial suspension was aerated, the bands b_1 and a_1 gradually disappeared, while the band a_2 at 630 mμ. was replaced by one at 645 mμ. When the aeration was stopped, the cytochrome bands reappeared in their reduced form. At room temperature and in the presence of an excess of substrate, the reduction was almost instantaneous. To follow the progressive changes in the absorption bands during this process, it was necessary to slow down the rate of reduction by cooling the bacteria. The tube containing the suspension of micro-organisms was placed in a small glass cylinder of 6 cm. diameter, the base of which was made of plane glass. The tube was then surrounded by ice. With this arrangement it was possible to increase the time for complete reduction to about 1 min., and to measure with precision the rate of reappearance of the bands of reduced cytochrome. Band b_1 reappeared first. As it became stronger, so the band a_2 at 645 mμ. faded and a shading appeared between 645 and 630 mμ. Finally, as b_1 reached its full intensity, the shading disappeared and a_2 returned to its original position (630 mμ). and intensity. In a typical experiment, the time for complete reduction was 105 sec.; the band b_1 reappeared after 30 sec. and the band a_2 began to move from 645 to 630 mμ. at about 35–40 sec. A comparison between the time of reduction of components b_1 and a_2 is usually complicated by the facts that their intensities are not the same, and that their absorption bands do not behave in the same way on oxidation: the band b_1 disappearing at 560 mμ., while a_2 at 630 mμ. is replaced by a band at 645 mμ. However, in the case of *A. aerogenes* used in this work, a_2 was almost as strong as b_1. The fact that on reduction the band b_1 reappeared before the strong band a_2 at 645 mμ. had shown any change in intensity or position, indicates that the component b_1 was certainly reduced before a_2. During aeration, however, the band a_2 moved very rapidly from 630 to 645 mμ., before any change in the intensity of b_1 could be observed. Thus the component a_2 was oxidized before b_1.

The absorption band a_1 was so faint that no definite conclusions about its reactions with the other components could be reached.

Since in all aeration experiments it became necessary to add octyl alcohol in order to prevent frothing, careful investigations were made to find out whether it had any action on the cytochrome or any other part of the respiratory system. Bacterial suspension (0·5 ml.) containing 5% glucose was cooled with ice under the spectroscope and about 0·001 ml. octyl alcohol was added. Under these conditions the time of complete reduction of the cytochrome bands, after aeration, was found to be 15 sec. The mixture was aerated intermittently over a period of 10 min., but the reduction time, after interrupting the air stream, remained constant at 15 sec. Octyl alcohol (0·04 ml.) was then added, and the time of reduction of the cytochrome bands again measured: it was found to be still 15 sec., and remained so for another 5 min. Finally a further 0·06 ml. of octyl alcohol was added. The reduction time immediately after this addition was still 15 sec., but the band a_2 seemed less sharp than before. However, 2 min. later, the time of reduction had increased to 20 sec., and after 2 hr. to about 4 min. In a second experiment, the bacterial suspension was treated with 0·02 ml. octyl alcohol at

room temperature, and then cooled with ice. Under these conditions, the reduction time was far greater than that of a control suspension which had received no octyl alcohol. Thus this reagent can safely be used in small amounts at 0° in studies of the oxidation and reduction of cytochrome, but is dangerous at higher temperatures. The effect of octyl alcohol was also studied manometrically (see below). Attempts to use tributyl citrate in place of octyl alcohol were unsuccessful. This substance strongly inhibited the reduction of cytochrome in *A. aerogenes*, even at 0°.

Azide (0·003 M) at room temperature has no observable effect on the cytochrome spectrum of the bacteria. At 0° different results were obtained after 2 months' interval on the same strain of *A. aerogenes*. Differences in the behaviour of the cytochrome bands were also noticed towards cyanide, at 2 months' interval (see below).

In a first series of experiments, when azide was added to a suspension of bacteria which showed the reduced cytochrome bands, no change of spectrum was observed. As soon as a few air bubbles had passed through the suspension, band a_2 moved from 630 to 645 mμ., i.e. to its oxidized position; but the band b_1, which in absence of azide disappears almost completely on oxidation, remained clearly visible at approximately half its original intensity, and could not be oxidized further by prolonged aeration. When the aeration was interrupted there was a marked delay in the return of the band a_2 to its normal reduced position at 630 mμ. In fact, the change was two to three times slower than was observed in absence of azide. However, it was no longer as intense as before. The band b_1 also did not return to its original strength. Hence, in the presence of azide, the band a_2 has a tendency to remain in the oxidized state, and the band b_1 to remain in the reduced state.

The action of azide was also studied in a suspension of bacteria in 50 % glycerol, frozen and cooled in liquid air. In the reduced state, azide did not bring about any change. To observe the oxidized state, the suspension was shaken vigorously in air, cooled to 0°, shaken again, and finally frozen and cooled in liquid air. Without addition of azide, the typical oxidized spectrum of *A. aerogenes* was seen. The very sharp b_1 band had almost completely disappeared, but the band a_2 was very sharp and strong at 645 mμ. If the same procedure was followed in the presence of azide, the band b_1 remained strong, but was slightly weaker than before, i.e. it was not fully reduced. The band of component a_2 was visible at 645 mμ., but since it was not as strong as in the azide-free control it follows that a_2 was not fully oxidized.

In a second series of experiments, carried out 2 months later, the above results could not be repeated: azide now had no effect on the absorption spectrum or on the rate of reduction of the cytochrome components. A new culture of the same strain of *A. aerogenes* I was obtained from the M.R.C. Unit for Chemical Microbiology, and cultivated under the standard conditions in broth. The resulting suspensions of bacteria showed the same reactions with azide as in the first series of experiments.

When pure carbon monoxide was bubbled through a bacterial suspension, in which the cytochromes were in the reduced state, the band a_2 moved from 630 to 635 mμ. and became slightly sharper. The bands b_1 and a_1 did not change.

Cyanide (0·002–0·001 M) did not modify the reduced cytochrome spectrum, although on aeration the band a_2 disappeared completely. By freezing the aerated suspension in liquid air, it was possible to see a faint band a_2 at the position of the reduced compound. After 5–8 min. at room temperature, the band a_2, which had disappeared on aeration, reappeared but did not return to its original intensity. These results could no longer be obtained when the behaviour of *A. aerogenes* had changed towards azide, in the course of 2 months: cyanide no longer affected the band a_2, although it still strongly inhibited the respiration at 30°.

Urethane (0·05 M) accelerated the oxidation, and slowed down the reduction of the cytochrome components, without modifying their intensities.

Hydrogen peroxide (0·02–0·1 M) at room temperature in presence of 0·0001 M-hydroxylamine, which effectively inhibits the catalase in *A. aerogenes*, had no effect on the absorption bands of cytochrome.

Cytochrome in metal-deficient bacteria

Washed metal-deficient bacteria were white in appearance, whereas those grown in nutrient broth were distinctly brownish grey. By using optimal conditions of illumination, and very thick suspensions of metal-deficient bacteria, the band b_1 could be clearly seen; it had about one-third the normal intensity. An extremely feeble band a_1 was just perceptible, but there was no detectable trace of a_2.

When the bacteria were mixed with glycerol and cooled in liquid air, the bands b_1 and a_1 were strongly reinforced, and it was possible to see a faint band a_2 at 630 mμ. Hence the intensity of band a_2 is much more dependent than that of a_1 or b_1 on the concentration of metals, of which iron is the most significant (Table 1). It should be noted that the band a_2 showed an extraordinary degree of variability, even in cultures grown under apparently identical conditions, while the intensities of the bands b_1 and a_1 remained remarkably constant.

Table 1. *The influence of the composition of the medium on the intensity of the absorption bands of cytochrome*

Media	Absorption bands a_2	a_1	b_1
Metal-deficient medium	Traces only	+	+ + +
Metal-deficient medium + iron	+ + +	+ +	+ + + + +
Synthetic medium + iron	+ + + +	+ +	+ + + + +
Nutrient broth	+ + + + +	+ +	+ + + + + +

Effect of azide on respiration of Aerobacter *cultivated in metal-rich medium*

The Q_{O_2} of the organism in presence of glucose at 30° was 110 and was independent of substrate concentration within the range 0·1–0·001 M. With pyruvate the optimal substrate concentration was 0·03 M, while with succinate it was 0·08 M, the Q_{O_2}'s being 74 and 26 respectively.

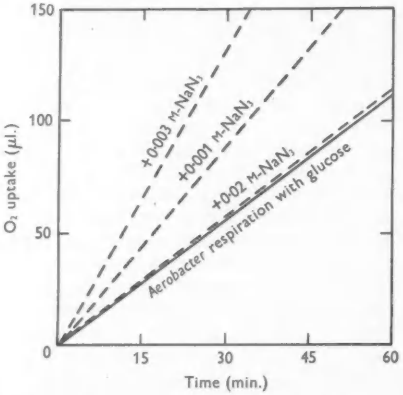

Fig. 1. The action of various concentrations of azide on *Aerobacter* respiration in presence of 0·01 M-glucose and 0·1 M-phosphate buffer pH 6·8 and with 1 mg. dry-weight bacteria.

With glucose as substrate, azide produced an increase in oxygen uptake, which was maximal (more than 100 %) at a concentration of 0·003 M (Fig. 1). This increased rate of oxygen uptake persisted for at least 3 hr. The oxidation of lactate or pyruvate was not markedly influenced by 0·003 M-azide at pH 6·8, whereas the oxidation of succinate and acetate was inhibited by about 65 %; it is known that the inhibition produced by azide varies with pH (Keilin, 1936), being stronger in more acid solutions. The effect of this substance on the oxidation of glucose, pyruvate and succinate was therefore investigated at various pH's (Fig. 2). The increased oxygen uptake brought about by azide in the presence of glucose was maximal at pH 7·3; at pH's 5·5 or 7·8 azide had no effect. With pyruvate, azide increased the oxygen uptake by 20 % at pH 7·3, but produced an inhibition of about 40 % at pH 5·5. With succinate, azide inhibited the oxygen uptake at all pH values between 5·5 and 7·8.

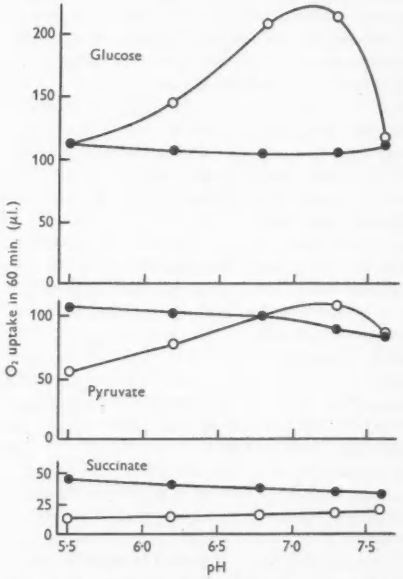

Fig. 2. The effect of azide on the oxidation of glucose, pyruvate and succinate, at various pH values, in presence of 0·1 M-phosphate buffer. ●—●, Values obtained without azide; ○—○, values obtained in the presence of 0·003-M-azide (1 mg. dry-weight bacteria).

Table 2. *The effect of azide on the oxygen uptake in presence of various sugars. The experiments were carried out at* 30° *in presence of* 0·1 M*-phosphate buffer*, pH 6·8

Substrates (0·01 M)	Q_{O_2} Without azide	+0·003 M-azide
Glucose	117	245
Fructose	119	116
Mannose	127	180
Galactose	51	54
Xylose	19	8
Dulcitol	14	7
Sorbose	14	14

The effect of azide was investigated in the presence of several sugars as shown in Table 2. The most marked increase (> 100 %) was obtained with glucose while mannose showed an increase of nearly 40 %. It is unlikely that this effect was due to glucose impurities. The oxygen uptake with fructose, which was equivalent to that of glucose, was not influenced by azide. With certain substrates which were slowly oxidized (xylose and dulcitol) azide exerted an appreciable inhibition.

Effect of other inhibitors on respiration

In order to obtain some information as to the mode of oxidation of glucose in the presence of azide, oxygen uptake, carbon dioxide output, glucose utilization and pyruvic and lactic acid formation, were estimated in the same experiment. The manometric technique was that of Dixon & Keilin (1933) which allows the direct determination of oxygen uptake and carbon dioxide output. Glucose was added from a dangling tube at the beginning of the experiment, and the reaction was stopped at the end of the experiment by tipping in 0·2 ml. 4 N-sulphuric acid from a side arm. Residual glucose, and pyruvic and lactic acids were estimated in trichloroacetic acid filtrates. The results of a typical experiment are shown in Table 3. In the absence of azide, the glucose carbon utilized during the experiment (24·36 μg.atoms) was recovered almost quantitatively as pyruvic and lactic acids and carbon dioxide (23·35 μg.atoms). In the presence of azide, 48·36 μg.atoms of carbon were utilized, but the carbon recovery was only 37·00 μg.atoms. It is possible that this discrepancy in the carbon balance was due to the formation of acetic acid, which was not estimated. In this experiment oxygen and glucose utilization and carbon dioxide production were all doubled by azide. Pyruvic acid production was increased by 40 %, whereas the yield of lactic acid dropped slightly. The quantity of pyruvic acid found during the oxidative breakdown of glucose was extremely high, both in the presence and in the absence of azide.

Table 3. *The action of azide on glucose breakdown*

(The experiment was carried out in presence of 0·1 M-phosphate buffer, pH 6·8, with 6·5 mg. dry-weight bacteria, during 30 min. at 30°, and with 12 μg.mol. of glucose as substrate. Results are given in μg.mol.)

	Without azide	With 0·003 M-azide
Oxygen taken up	8·25	17
Glucose utilized	4·06	8·06
Pyruvic acid formed	4·7	6·7
Lactic acid formed	1	0·8
Carbon dioxide output	6·25	14·5
R.Q.	0·76	0·85

It was thought that the increased oxygen uptake brought about by azide might be due to a direct oxidation of glucose by an enzyme similar to glucose oxidase, with the production of hydrogen peroxide. The azide-catalase test, as described by Keilin & Hartree (1945), was used for the detection of hydrogen peroxide. A suspension of bacteria with 0·1 M-glucose and 0·1 M-phosphate buffer, pH 6·5, was placed under the microspectroscope and aerated vigorously for 30 min. Azide-catalase was then added, but the characteristic 'reduced azide-catalase', which is produced specifically by hydrogen peroxide, could not be detected. Neither was it formed if azide was present during the aeration. Attempts to show the presence of hydrogen peroxide in the clear supernatant after centrifuging down the bacteria were also unsuccessful, in presence of either 0·0001 M-hydroxylamine, which inhibits catalase, or azide.

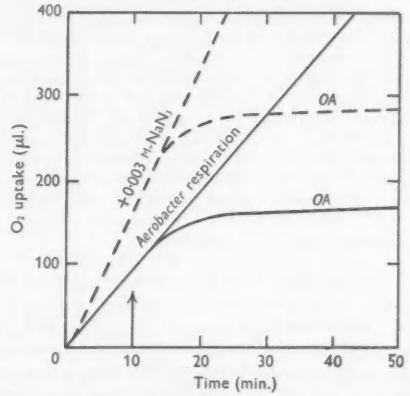

Fig. 3. The action of octyl alcohol (OA) on the respiration of *Aerobacter* at 30°, in the presence or in the absence of azide, and with glucose as substrate and 0·1 M-phosphate buffer pH 6·8. Octyl alcohol (0·03 ml.) was added in the manometric flask at the time indicated by the arrow.

At 30°, it was found that 0·02–0·05 ml. octyl alcohol in 3·0 ml. bacterial suspension completely inhibited the oxygen uptake of *A. aerogenes* with glucose, either in the absence or in the presence of azide (Fig. 3). As these results were not in agreement with spectroscopic experiments carried out at 0°, the manometric experiments were repeated at the latter temperature. Under such conditions 0·02–0·05 ml. octyl alcohol did not produce any inhibition of the oxygen uptake, either in the absence, or in the presence of azide (Fig. 4), and furthermore azide alone gave only a very slight increase in the oxygen uptake.

A mixture of carbon monoxide (95 %) with oxygen (5 %) produced an inhibition of about 14 % in the

respiration of *A. aerogenes* in presence of glucose, but 90% carbon monoxide with 10% oxygen had no effect. In these experiments the control vessels contained nitrogen in place of carbon monoxide.

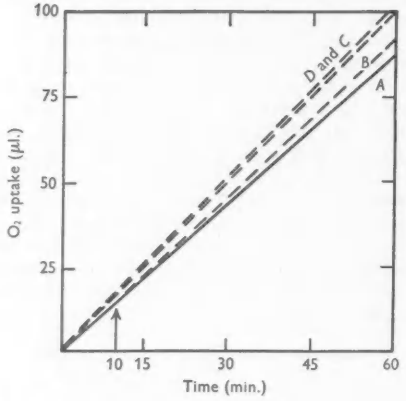

Fig. 4. The action of octyl alcohol on the respiration of *Aerobacter* at 0°, in the presence and in the absence of azide, and with glucose as substrate and 0·1 M-phosphate buffer pH 6·8. Octyl alcohol (0·03 ml.) was added to the manometric flask at the time indicated by the arrow. Curve *A*, *Aerobacter* respiration; curve *B*, *Aerobacter* respiration + octyl alcohol; curve *C*, *Aerobacter* respiration + octyl alcohol + 0·003 M-azide; curve *D*, *Aerobacter* respiration + 0·03 M-azide.

Potassium cyanide (0·001 M) inhibited the oxygen uptake with glucose, pyruvate or succinate by about 95%, while 0·0001 M-cyanide produced 50% inhibition. This effect could be reversed by adding a small amount of methaemoglobin from a dangling tube into the manometric flask. Methaemoglobin has a very great affinity for cyanide with which it forms the stable cyan-methaemoglobin (Haurowitz, 1931).

Effect of inhibitors on respiration of metal-deficient bacteria

With glucose as substrate, bacteria cultivated in metal-deficient media were found to have an oxygen uptake similar to that of bacteria grown (*a*) in a metal-deficient medium to which had been added 40 μg. iron/l. in the form of ferrous ammonium sulphate or (*b*) in nutrient broth. The oxidation of pyruvate was found to be decreased by 20% with metal-deficient *Aerobacter* and that of acetate and succinate by 60–65%. Bacteria grown on the deficient medium with added iron (40 μg./l.) oxidized these substrates at the normal rate.

Sodium azide (0·001 M) increased the oxygen uptake with glucose as substrate at pH 6·8 as it did with normal cells, but at pH 5·5, although it increased the oxygen uptake by about 65% with normal bacteria, it produced at first a small increase in oxygen uptake and then almost completely inhibited the respiration. Carbon monoxide (90%) with oxygen (10%), which did not affect the respiration of cells grown under normal conditions, inhibited the oxygen uptake of metal-deficient bacteria by 50–60%. With a carbon monoxide: oxygen ratio of 19:1 an inhibition of about 65% was obtained. This inhibition could not be reversed by light, although the intensity of the mercury vapour lamp used was sufficient to abolish the carbon monoxide inhibition of the respiration of baker's yeast. The respiration of metal-deficient bacteria was found to be much more sensitive to small concentrations of cyanide than that of bacteria grown under normal conditions: 0·0001 M-cyanide, which only produced 50% inhibition with normal cells, inhibited the oxygen uptake of metal-deficient *Aerobacter* by 90%; 2:2′-dipyridyl and 8-hydroxyquinoline (0·005 M) increased the oxygen uptake by about 25% with both normal and metal-deficient cells while 0·001 M-phenylthiourea produced a similar but smaller effect.

Dehydrogenase activity in Aerobacter *grown in metal-rich medium*

The results of experiments on the anaerobic reduction of methylene blue are summarized in Tables 4–6. They show: (1) that with glucose as substrate the rate of reduction was nearly four times faster in the presence of 0·003 M azide; (2) that azide inhibited the reduction by more than 50% in the presence of succinate or acetate; and (3) that in the presence of fructose or galactose azide had practically no effect, while with mannose the reduction was stimulated by azide although not as much as with glucose.

Octyl alcohol greatly increased the rate of reduction of methylene blue with glucose as substrate, both at 30 and at 0° (Table 7). As the respiration was completely inhibited in a Barcroft manometer where regular shaking ensured the dispersion of octyl alcohol, it was important to repeat the reduction experiments, shaking the Thunberg tubes in the thermostat. However, even under these conditions, the methylene-blue reaction was still as markedly increased as in the experiments where the tubes were stationary.

Dehydrogenase activity in metal-deficient bacteria

The rate of methylene-blue reduction in presence of glucose was not markedly altered by metal deficiency. But with succinate and acetate the reaction rate was slowed down to about one-third, and with formate the reaction was almost completely abolished (Table 8).

DISCUSSION

The properties of the component a_2 in *A. aerogenes* are similar to those of cytochrome a_2, which is generally believed to be identical with cytochrome oxidase (Keilin & Hartree, 1939); thus a_2, like a_3, reacts with carbon monoxide and cyanide. Moreover, by following the reduction and the oxidation of the cytochrome bands under the microspectroscope, it has been observed that the band a_2 is reduced after b_1 and oxidized before it. These two arguments strongly support the view that cytochrome a_2 functions as cytochrome oxidase.

The variations in intensity of the a_2 band, and also in its general aspect, are marked in different species. Thus, the bands a_2 in *Azotobacter* ($Q_{O_2} = 5000$) and in *Acetobacter* ($Q_{O_2} = 1000$) are much weaker and narrower than in *Aerobacter* ($Q_{O_2} = 100$). It follows that there is no relation between the Q_{O_2} and the strength of the band, but it is possible that the catalytic activity of the component a_2 varies in different species.

Table 4. *The action of different concentrations of azide on the anaerobic reduction of methylene blue in the presence of* 0·01 M*-glucose,* 0·1 M*-phosphate buffer* pH 6·8, *at* 30°

Time of reduction of methylene blue (sec.)			
	With azide		
Without azide	0·003 M	0·01 M	0·02 M
300	80	90	120

Table 5. *The action of* 0·003 M*-azide on the reduction of methylene blue with glucose, succinate and acetate, at* 30°

(0·1 M-phosphate buffer, pH 6·8.)

Substrates	Time of reduction of methylene blue (sec.) Without azide	With azide
Glucose (0·01 M)	1320	360
Succinate (0·04 M)	900	2100
Acetate (0·04 M)	900	1920

In metal-deficient bacteria, the concentration of cytochrome a_2 is reduced to a minute trace (only perceptible after cooling the bacteria to the temperature of liquid air), whereas cytochromes b_1 and a_1 are reduced by only 50–70 %. If the component a_2 is the only cytochrome component which reacts directly with molecular oxygen, and is thus one of the indispensable steps in the respiration of this organism, it is difficult to understand why, in iron-deficient cells, its concentration is decreased more markedly than that of the other cytochrome components, while at the same time the rate of oxygen uptake is unchanged. These facts point rather to the conclusion that there is yet another autoxidizable catalyst present which links the

Table 6. *The action of* 0·003 M*-azide on the methylene blue reduction in presence of various sugars, at* 30°, *and with* 0·1 M*-phosphate buffer* pH 6·8

Substrates (0·01 M)	Time of reduction of methylene blue (sec.) Without azide	With azide
Glucose	170	50
Fructose	165	145
Mannose	180	105
Galactose	190	170
Xylose	900	>1800

Table 7. *The effect of octyl alcohol (OA) on the reduction of methylene blue in presence of* 0·1 M*-phosphate buffer,* pH 6·8, *without and with the addition of azide, and with glucose* (0·01 M) *as substrate*

Temp. (°)	Time of reduction of methylene blue (sec.) Without azide		+0·003 M-azide	
		+0·002 ml. OA		+0·02 ml. OA
30	420	55	120	45
		+0·1 ml. OA		+0·1 ml. OA
30	420	30	120	25
		+0·002 ml. OA		+0·02 ml. OA
0	1620	540	1380	660

Table 8. *The effect of iron deficiency on the reduction of methylene blue with glucose, succinate, acetate and formate*

(6 mg. dry-weight bacteria were used per Thunberg tube (0·1 M-phosphate buffer, pH 6·8).)

Substrate (0·01 M)	Time of reduction of methylene blue (sec.) Bacteria grown in normal medium, or in metal-deficient medium + iron	Metal-deficient bacteria
Glucose	180	200
Succinate	170	515
Acetate	165	550
Formate	300	>1800

cytochrome system with oxygen. Since in metal-deficient cells the sensitivity of the oxygen uptake towards cyanide and especially towards carbon monoxide is greatly increased, it is safe to assume that the supposed oxidase is a metal protein, the metal of which would most likely be iron or possibly copper. It is not an unreasonable supposition that component a_1 is this autoxidizable catalyst, for, under certain circumstances, it has been observed to react with carbon monoxide and cyanide (Warburg & Negelein, 1933). But it is also possible that the oxidase is of a type which is not detectable by direct spectroscopic examination of the cells. There is, however, an alternative possibility which must be considered: normal cells contain a great excess of a_2 and even if the amount of this catalyst is very much reduced, it is still sufficient to insure normal respiration. This would explain the increased sensitivity of metal-deficient cells towards inhibitors.

The inhibition of the respiration of *A. aerogenes* by cyanide is due to its combination with cytochrome a_2, and perhaps with some other metal catalysts. But it is now obvious that the inhibition of respiration is not due to any effect on catalase, as was supposed by Aubel, Rosenberg & Szulmajster (1950) in the case of *Escherichia coli*, since azide, a powerful inhibitor of catalase, is without effect.

Azide did not inhibit the respiration of *A. aerogenes* in the presence of glucose or other sugars; but when succinate or acetate were used as substrates, azide (0·003 M) inhibited the rate of oxygen uptake by more than 60 %. Aubel & Szulmajster (1947) reported similar results with *Esch. coli*. One might suppose that the oxidation of glucose did not proceed through the same final chain of reactions as did succinate or acetate. However, with these two latter substrates both the rate of reduction of methylene blue and the oxidation measured manometrically were inhibited by azide (0·003 M) to the same extent. It is thus very likely that the same catalytic system was inhibited in each experiment which indicates that the inhibition does not take place at the final steps of the aerobic oxidation but rather at the dehydrogenase level.

Metal deficiency, like azide, produced a decrease of about 60 % in both the methylene-blue reduction and the oxygen uptake, with succinate or acetate as substrates. As this effect could be abolished by addition of iron only to the culture medium, an iron catalyst, concerned in the oxidation of these two substrates, is apparently involved at the dehydrogenase level.

At room temperature, where the rate of reduction of the cytochromes is very rapid, no action of azide on the absorption bands, or on the rate of their reduction, could be detected spectroscopically. At 0°, on the other hand, where the rate of reduction was considerably slower, azide was seen to decrease this rate. However, this action is far less marked than that which may be observed for example in the cytochrome oxidase system of heart-muscle preparations in which azide completely inhibits the oxidation of all cytochrome components. Moreover, the feeble action of azide on cytochrome reduction in *A. aerogenes* disappeared as a result of repeated subculturing over a period of 2 months.

Azide (0·003 M) doubled the quantity of glucose metabolized, the oxygen taken up and the carbon dioxide produced. The quantity of pyruvic acid formed was increased by 40 % while that of lactic acid dropped slightly. This action of azide was found to be specific for glucose: with fructose and galactose, two sugars which were oxidized at the same rate as glucose, azide did not produce any increase in oxygen uptake or in the rate of methylene blue reduction. At neutral pH, azide had no marked action on the oxidation of pyruvate, a substance which was found in very large amounts during glucose oxidation, and was presumably an essential intermediate in the aerobic glucose breakdown. At acid pH, pyruvic oxidation was inhibited by azide, while the oxygen uptake with glucose was either increased or not affected. Thus it can be concluded that azide exerts two opposing actions on the oxidation of glucose at acid pH: a stimulation during the first steps of the oxidation leading to pyruvate, followed by inhibition during pyruvate breakdown.

Although the oxidation of glucose is stimulated by azide when either oxygen or methylene blue are used as final oxygen acceptors, the anaerobic fermentation of glucose is not affected by azide. Oxygen or some other hydrogen acceptor appears to be indispensable.

Aubel *et al.* (1950), studying the action of azide and other inhibitors on the respiration of *Esch. coli*, concluded that the oxidation of glucose and pyruvate did not go through the tricarboxylic acid cycle. Their view was based mainly on the facts that azide, malonate and fluoride, which produced little or no inhibition of glucose and pyruvate oxidation, strongly decreased the rate of oxidation of succinate and fumarate. In the case of *A. aerogenes* used in the course of the present work, the oxidation of pyruvate at neutral pH was stimulated by azide, whereas that of succinate was inhibited. However, this does not in itself prove that succinate is not an intermediate in pyruvate breakdown. Thus at acid pH the oxidation of pyruvate was inhibited while that of glucose was unchanged or even stimulated. Nevertheless, there is some evidence that pyruvate is an intermediate in glucose breakdown. It should be borne in mind that the inhibition by azide of the oxidation of succinate or acetate did not, in the present experiments, exceed 65 %, and that the residual activity might well be adequate to bring about the

oxidation of these substances, should they be formed as intermediates.

The specific action of azide on glucose suggested that a special oxidizing enzyme, of the glucose oxidase type (Keilin & Hartree, 1948), might be concerned. However, hydrogen peroxide could not be detected during the oxidation of glucose by the bacteria.

Clifton (1937), Clifton & Logan (1939), and others found that azide prevented the assimilation of the substrate to build up cellular material, thus permitting the oxidation of the substrate to proceed to completion. The oxygen uptake was not markedly increased at the beginning of their experiments, but it remained constant and eventually accounted for complete oxidation of the substrate. However, in the experiments done in the course of the present work, azide increased the rate of respiration as soon as it came into contact with the bacteria.

A new mode of action of azide has been found in the last few years: Hotchkiss (1944) and Spiegelman, Kamen & Dunn (1946) mentioned that azide prevented aerobic phosphate uptake in yeast, while Spiegelman, Kamen & Sussman (1948) presented some evidence that triosephosphate dehydrogenase was concerned. Recently, Loomis & Lipmann (1949), working with a kidney enzyme preparation and glutamate as substrate, also found that azide prevented the esterification of inorganic phosphate. If azide reduces the formation of energy rich phosphate, one might think that it leads to an increased rate of substrate utilization, to provide the cells with the necessary energy. However, in the experiments of Spiegelman *et al.* (1948), as in those of Loomis & Lipmann (1949), no marked increase in the rate of substrate breakdown was observed.

SUMMARY

1. *Aerobacter aerogenes*, grown in a normal medium (nutrient broth), shows the absorption bands of reduced cytochrome occupying the following positions: a_2, 630 mμ.; a_1, 590 mμ.; and b_1, 560 mμ. On oxidation, the absorption bands a_1 and b_1 disappear, while a_2 moves from 630 to 645 mμ.

2. The concentration of cytochrome a_2 in *Aerobacter* is higher than in any other microorganism so far examined, the band a_2 being almost as strong as b_1. The band a_1 is very faint.

3. On aeration, cytochrome a_2 is oxidized before b_1, and by stopping the aeration, it is reduced after b_1. Moreover, cytochrome a_2 combines with carbon monoxide and cyanide. These two facts support the view that in *A. aerogenes* a_2 acts as cytochrome oxidase.

4. In *Aerobacter* grown in metal-deficient medium the band a_2 becomes hardly detectable, whereas the intensities of a_1 and b_1 are reduced to one-half or one-third of their usual intensities.

5. The respiration in presence of glucose is not affected by metal deficiency.

6. If normally cytochrome a_2 is present in great excess, it might still, in metal-deficient cells, be present in sufficient amount to allow normal respiration.

7. Azide does not combine with any of the cytochrome components of *A. aerogenes*.

8. Azide (0·003 M) increases the oxygen uptake with glucose by more than 100 %, and the anaerobic reduction of methylene blue by 300–400 % at 30°. This effect is very feeble at 0°.

9. Azide (0·003 M) increases the rate of glucose utilization and that of carbon dioxide production by about 100 %, and the amount of pyruvate found at the end of the experiment by 40 %.

10. Azide has no effect on the respiration or on the methylene-blue reduction in presence of fructose or galactose.

11. Azide (0·003 M) inhibits by about 60 % the oxygen uptake and the rate of methylene-blue reduction in presence of succinate or acetate.

12. The oxygen uptake and the methylene-blue reduction by metal-deficient cells is reduced by about 60 % with succinate and acetate as substrate. Since the addition of iron to the metal-deficient medium is sufficient to abolish this effect, it follows that an iron catalyst, necessary for the anaerobic reduction of methylene blue, is apparently involved in the oxidation of succinate and acetate.

13. Octyl alcohol (0·02 ml. in 3 ml. bacterial suspension) completely inhibits the oxygen uptake in the presence of glucose and increases the rate of methylene-blue reduction by eight to ten times at 30°, with the same substrate.

14. At 0° the same amount of octyl alcohol has no effect on the oxygen uptake, but it still increases the rate of methylene-blue reduction by more than 100 %, with glucose as substrate.

15. Octyl alcohol (0·001–0·01 ml. in 0·5 ml. bacterial suspension) slows down the rate of reduction of the cytochrome components at room temperature, but has no effect at 0°.

16. Hydrogen peroxide could not be detected during the respiration of *A. aerogenes* in presence of glucose.

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